UNIVERSITY OF HAWAII
LIBRARY
OCT 7 '52

EXPERIMENTAL CELL RESEARCH

E
CR

Published under the auspices of

THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

EDITORS:

TORBJÖRN CASPERSSON · HONOR B. FELL · JOHN RUNNSTRÖM

FRANCIS O. SCHMITT · PAUL WEISS · RALPH W. G. WYCKOFF

VOLUME 3 · SEPTEMBER 1952 · NUMBER 3

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N.Y.

EXPERIMENTAL CELL RESEARCH

EDITORS:

T. Caspersson, Department of Cell Research, Karolinska Inst., Stockholm 60, Sweden
Honor B. Fell, Strangeways Laboratory, Cambridge, England
J. Runnström, The Wenner-Gren Institute for Experimental Biology, Stockholm, Sweden
Francis O. Schmitt, Massachusetts Institute of Technology, Cambridge 39, Massachusetts
Paul Weiss, University of Chicago, Chicago 37, Illinois
Ralph W. G. Wyckoff, Laboratory of Physical Biology, National Institute of Health, Bethesda, Maryland.

EXECUTIVE COMMITTEE:

T. Caspersson, Honor B. Fell, J. Runnström and J. F. Danielli, King's College, London, W. C. 2, England.

Papers originating from Europe should be submitted to Dr. Honor B. Fell or to the Editorial Office, Norrtullsgatan 16, Stockholm Va., Sweden.

Papers from the United States and South America should be sent to one of the American Editors.

ADVISORY BOARD:

H. Bauer (Wilhelmshafen), W. Bloom (Chicago), J. Brachet (Bruxelles), F. Buchthal (Copenhagen), A. Buzzati-Traverso (Verbania Pallanza), R. Chambers (New York), E. Chargaff (New York), J. F. Danielli (London), C. D. Darlington (London), J. N. Davidson (Glasgow), M. Demerec (Cold Spring Harbor, N. Y.), J. Engelbreth-Holm (Copenhagen), E. Fauré-Frémiet (Paris), A. Fischer (Copenhagen), A. Frey-Wyssling (Zürich), N. Fries (Uppsala), R. J. Gautheret (Paris), L. Geitler (Wien), G. Gomori (Chicago), A. Haddow (London), E. Hammarsten (Stockholm), B. Hanström (Lund), E. Newton Harvey (Princeton, N. J.), L. V. Heilbrunn (Philadelphia, Pa.), G. C. Heringa (Amsterdam), A. Hollaender (Oak Ridge, Tenn.), H. Holter (Copenhagen), J. Holtfreter (Rochester, N. Y.), P. Koller (London), F. Lehmann (Bern), P. L'Héritier (Paris), G. Levi (Torino), P. E. Lindahl (Uppsala), G. Lindeberg (Uppsala), C. C. Lindegren (Carbondale, Ill.), R. J. Ludford (London), S. E. Luria (Bloomington, Ind.), A. Lwoff (Paris), A.Mirsky (New York), G. Montalenti (Naples), H. Okkels (Copenhagen), O. Olivo (Bologna), A. W. Pollister (New York), J. Randall (London), W. J. Schmidt (Giessen), F. Schrader (New York), W. Seifriz (Philadelphia, Pa.), T. M. Sonneborn (Bloomington, Ind.), J. M. Sosa (Montevideo), J. Spek (Rostock), W. M. Stanley (Berkeley, Calif.), E. Suomalainen (Helsingfors), E. L. Tatum (Stanford University, Calif.), T. Teorell (Uppsala), A. Tyler (Pasadena, Calif.), W. Wilbrandt (Bern).

Subscriptions

EXPERIMENTAL CELL RESEARCH

will be published in four issues annually at $15.00 per volume. The price of one issue is $4.00. Please send your subscription orders to Academic Press Inc., 125 East 23 St., New York 10, N.Y.

STUDIES ON MITOCHONDRIA:

V. THE RELATIONSHIP OF STRUCTURE AND OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA OF HEART MUSCLE[1]

JOHN W. HARMAN and MURIEL FEIGELSON

Department of Pathology, Medical School, University of Wisconsin, Madison, Wisc.

Received August 20, 1951

EARLY studies of oxidative enzyme systems indicated that the integrity of some component or structural relationship in the particulates of disrupted cells was required for efficient oxidative catalysis (14, 29). Several explanations were offered, and it was suggested that the organization of the system operated at cellular (11), colloidal (29), physicochemical (2) and molecular (38) levels. It was agreed upon, however, that the high activity of these systems was due to the maintenance within the extracted cellular fragments of the favorable spatial organization of the enzymes peculiar to the cell. Subsequent investigations elicited more direct experimental proof for the existence of the integrated enzyme complex (15, 31, 43) which implemented the oxidation of carbohydrate, fats and amino acids. It was demonstrated that the respiratory function of this complex was dependent on the presence and integrity of the mitochondria (17, 22), which manifested *in vitro* a very precise relationship between their form and oxidative capacity (18, 19). Thus was defined a connection between organization of recognized cellular constituents and the respiratory capacity.

The more recent studies on the alterations of oxidation by agents which uncouple oxidation and phosphorylation (7, 8, 21, 47) suggested that the structural organization of the particles or tissues studied considerably influenced the process of oxidative phosphorylation. In view of this and the previously discussed connection between oxidation and structure, it seemed pertinent to inquire into the relation of phosphorylation and the structure of the mitochondria. This possibility has been explored in the present study. The facts accumulated have been evaluated in relation to the dynamics of cellular respiration, with special regard to the role of the mitochondria in cellular metabolism.

---

[1] This investigation was supported by a grant from the Life Insurance Medical Research Fund.

METHODS

1. *Preparation of mitochondria.* Rabbit heart mitochondrial suspensions were prepared by homogenization of the tissue and differential centrifugation in a sucrose medium, in essentially the same manner as previously described (18), except for several minor variations. All centrifugations were carried out in a refrigerated International Centrifuge, Type SB-1. The initial centrifugation of the 0.25 M sucrose homogenate was carried out at 1 150 r.p.m., or a force of 275 × g in a horizontal free-swinging head for 5 minutes. Subsequent centrifugations of the supernate to precipitate and wash the mitochondria were carried out in an angle head at an r.p.m. of 4 100, or a force of 3 000 × g for 10 minutes each.[1] In order to increase the yield of mitochondria, in some of the preparations the first residue, consisting of incompletely ruptured cells, myofibrils, nuclei and numerous mitochondria, was resuspended in a small volume of homogenizing fluid, homogenized in a Potter-Elvehjem homogenizer (38) and centrifuged for 5 minutes at 1 150 r.p.m. at a force of 275 × g in a horizontal head. The supernatant obtained from this operation was combined with the supernatant from the original centrifugation of the homogenate, and the subsequent procedures used to precipitate, wash and suspend the mitochondria were continued as previously described. This modification was not routinely employed, since it involved an increase in the time of preparation. The final volume of the mitochondrial suspension was kept to the minimum required for each experiment, usually 5 to 6 ml.

Cyclophorase preparations were made as previously reported (17) except that 0.25 M sucrose instead of 0.9 per cent KCl was used as the homogenizing, washing and suspending medium.

2. *Oxidation and phosphorylation measurements.* Warburg manometric procedures were employed to estimate oxygen uptake during the oxidation of α-ketoglutarate, as described in a previous communication (18) where the basic components and maintenance of tonicity in the flasks have been presented. In some cases, however, as indicated, potassium adenosine-triphosphate (ATP) prepared from the dibarium salt (obtained from Sigma Chemical Co. and Nutritional Biochemicals Corp.) was substituted for adenosine-5-phosphate (AMP). Where indicated in the tables, the following agents which uncouple oxidation and phosphorylation have been added to the flasks: 2,4-dinitrophenol (Eastman Kodak Co.), James Green B (Diazine Green) (C. I. No. 133) (National Aniline Division, Allied Chemical and Dye Co.) and usnic acid.[2] That 2,4-dinitrophenol, Janus Green B and usnic acid uncouple oxidation and phosphorylation has been shown by the following authors, respectively: Hotchkiss (24), Judah and Williams-Ashman (27), and Johnson, Feldott and Lardy (26). The uncouplers were used at the concentrations recommended by these workers as

[1] Due to increased viscosity when 0.5 M sucrose was used as the homogenizing and washing fluid, the initial slow centrifugation time at 275 × g was increased to 8 minutes, and the subsequent centrifugation times at 3 000 × g were increased to 15 minutes each. These increases in centrifugation times produced a degree of sedimentation comparable to that obtained with 0.25 M sucrose at the shorter time.

[2] We are indebted to Dr. Henry A. Lardy of the Enzyme Institute, University of Wisconsin, who kindly supplied us with a sample of usnic acid.

optimal for the uncoupling oxidation and phosphorylation. The duration of the oxidation period was 20 minutes, unless otherwise indicated.

When P/O ratios were determined during oxidative phosphorylation glucose and hexokinase were employed to trap the high energy phosphate (~ P) as glucose-6-phosphate. The hexokinase[1] was prepared from yeast according to the method of Berger, Slein, Colowick and Cori (5), the isolation procedure being carried out as far as fraction 3 a, which was then lyophilized. Quantities of hexokinase were used which were found to give the highest P/O ratios for the system employed. The glucose and hexokinase solutions were added to the side arms of the Warburg flasks, along with the α-ketoglutarate and were tipped into the main chamber at the end of the 8 minute thermal equilibration period. Except where the effect of the hexokinase system on the morphology of the mitochondria was specifically under study, the hexokinase and glucose were omitted from those flasks used for cytological examination and added only to those flasks on which phosphate estimations were to be performed. Two sets of flasks to demonstrate each phenomenon were therefore run, one containing hexokinase and glucose for phosphate determinations and one omitting hexokinase and glucose for cytological examination. Fluoride was added to all flasks (cytological and phosphorylation) in experiments where P/O ratios were determined, in order to minimize adenosine-triphosphatase activity (39).

In several experiments the creatine-creatine transphosphorylase system was used to trap the high energy phosphate as creatine-phosphate. Twenty mg of creatine were added to each flask by pipetting 2 ml of a 1 per cent creatine solution into each flask and placing the flasks in a 100° C oven until the water had evaporated. In all experiments where creatine was not used the flask components other than sucrose, since they contributed little to the osmotic pressure, were neglected in computing tonicities. However, because such large quantities of creatine were used in the creatine experiments, the osmotic pressure contributed to the solution by the creatine was included in the computations of tonicity. Since the creatine trapping system had no effect on the morphology of the mitochondria, the creatine was added to both the cytological and phosphorylation flasks.

In experiments where P/O ratios were determined, the mitochondria were allowed to respire for 20 minutes at 37° C, at which time they were rapidly removed from the Warburg bath and placed in chipped ice. Two ml of cold, 10 per cent trichloracetic acid were immediately added to each flask. The precipitates were centrifuged down, discarded and the supernatants analyzed for inorganic phosphorous by the method of Fiske and Subbarow (12) when the hexokinase system was used, and by that of Lowry and Lopez (34) when the creatine system was used. The quantity of inorganic phosphorous esterified was computed by subtracting the inorganic phosphate values obtained from the value obtained on a zero-time sample (trichloracetic acid added before the addition of the mitochondrial suspension). The "labile P ester" was estimated in the trichloracetic filtrates by 7 minute hydrolysis with N HCl at 100°C. The phosphate ester not hydrolyzed by the Lowry-Lopez method, but hydrolyzed by the Fiske-Subbarow method is considered to be phospho-creatine. The ester not

---

[1] We are indebted to Dr. John H. Copenhaver, Jr., of the Enzyme Institute, University of Wisconsin, who kindly supplied us with one of the samples of hexokinase prepared in the manner herein described.

hydrolyzed by the Fiske-Subbarow method but hydrolyzed during the 7 minute hydrolysis period is designated "labile P ester." The ester not hydrolyzed during the 7 minute hydrolysis period is termed "stable ester."

In recording the oxygen uptake and phosphate esterification data, except as otherwise indicated, values obtained on the "blanks" (flasks in which α-ketoglutarate was omitted) have been subtracted from values obtained on the "experimentals" (flasks in which α-ketoglutarate was included).

Estimations of adenosine-triphosphatase activity have been performed by the method of DuBois and Potter (10). The reaction mixture, containing sodium barbital buffer, adenosine-triphosphate and $CaCl_2$ in the desired concentrations of sucrose and KCl was thermally equilibrated at 37° C in a Dubnoff metabolic shaking incubator for 5 minutes. The aliquot of mitochondrial suspension was then added and the reaction allowed to proceed for 15 minutes at 37°, at which time 0.3 ml of 50 per cent trichloracetic acid was added. Fiske-Subbarow inorganic phosphate determinations were performed on each sample. The values reported have been subtracted from zero-time controls and have been corrected also for controls in which the ATP and the mitochondrial suspension, respectively, have been omitted.

3. *Cytological technique.* The cytological examination of the mitochondria in the Warburg flasks reserved for this purpose was made in the manner previously described (18). Differential mitochondria counts were made with the aid of the phase microscope, Dark M Contrast, on samples removed from the Warburg bath at the end of the oxidation period. Detailed descriptions and photomicrographs of the various types of mitochondria have been presented in previous communications (18, 19). Except in Table II a, differential counts on "experimental" flasks only have been presented in the tables, counts on "blank" flasks having been omitted from the tabulation since they have invariably paralleled those obtained on "experimentals."

During the preparation of the mitochondrial suspensions each definitive centrifugal step was checked cytologically to determine the efficiency of the separation of the mitochondria from the myofibrils and nuclei. This routine examination was necessary if reliable, uniformly clean preparations were desired, because some of the shorter myofibrils and the unruptured nuclei tended occasionally to remain suspended in the mitochondrial supernate. When the cytological study revealed such a condition, an additional short spin of 3 minutes in the horizontal head as previously described reduced the contamination effectively. It is pertinent that the heart nuclei are unruptured by the homogenization with the Waring blendor and thus behave differently than the nuclei of other tissues comminuted by this instrument (17).

RESULTS

The variation in the osmotic pressure of the sucrose media suspending the mitochondria alters the rate of oxidation and the extent of oxidative phosphorylation significantly. As was observed in a previous study (18), the optimal osmolar concentration of sucrose was between 0.125 M and 0.250 M sucrose, when the oxygen uptake was maximal. This range of osmolarity

TABLE I

The effect of sucrose molarity on morphology, oxidation and phosphorylation of mitochondria.

Experiment	Sucrose molarity	Morphology (per cent)			Oxidation and phosphorylation		
		"Target"	"Spherical-dense"	"Limbus" and "crescent"	μ liters O_2 uptake	μ moles inorganic P esterified	P/O
A	0.500	16	67	17	17.6	3.4	2.16
	0.250	42	40	18	39.5	9.6	2.72
B	0.500	46	40	14	15.5	2.2	1.60
	0.250	62	27	11	35.0	7.0	2.23
C	0.250	62	25	13	12.4	2.6	2.34
	0.125	81	12	7	17.5	3.4	2.19
	0.055	67	16	17	15.9	2.1	1.48

TABLE I. In experiments A and B the mitochondria were prepared in 0.5 M sucrose; in experiment C they were prepared in 0.25 M sucrose. 0.5 ml of mitochondrial suspension was added to the Warburg flasks. The flasks were made up to a final volume of 2 ml and contained per ml of fluid the following additions: 0.5 μ moles of sodium succinate; 1.5 μ moles of adenosine-5-phosphate, 10 μ moles of potassium phosphate buffer, pH 7.25; 3 μ moles of sodium α-ketoglutarate (added to the side arm of "experimental" flasks); 10 μ moles of $MgCl_2$; 11.6 μ moles of NaF; 15 μ moles of glucose, and 1.5 mg of lyophilized hexokinase. The glucose and hexokinase were added only to the side arms of the phosphorylation flasks and were omitted from the cytological flasks. The final sucrose concentration in each flask was adjusted to the values indicated by adding appropriate quantities of a sucrose solution of suitable concentration. The filter paper in the center well was saturated with 0.2 ml of 5 N NaOH. The gaseous phase was oxygen. The flasks were thermally equilibrated at 37° C for 8 minutes, at which time the side arm contents were tipped into the main chamber.

is associated with the highest level of phosphorylation (Table I) by the hexokinase trapping mechanism. It is apparent from the concomitant cytological studies of mitochondrial form that the mitochondria, at this osmolarity, are predominantly active "target" mitochondria, although the resting particles prior to their addition into the complete experimental mixture contain few such forms and are mainly "spherical-dense." Departure from this osmolarity by either dilution or increased concentration depresses both oxidation and phosphorylation and establishes a lower percentage of "target" mitochondria, with a proportionate increase in the "crescent" type, which is a degenerative change (18). The fall in oxidative phosphorylation exceeds the drop in oxidation in most instances, so that there is usually a simultaneous decrease of the P/O ratio. The osmolarity of the medium, therefore, affects the oxidation and the phosphorylation of the mitochondria to different extents. Although it is indicative that the degenerative alterations of the mito-

TABLE II a

The effect of fluoride on morphology and oxidation.

Experiment	Preparation	Fluoride	Morphology						μ liters O_2 uptake	Oxidation period (minutes)
			"Blank"			"Experimental"				
			"Target"	"Spherical-dense"	"Limbus" and "crescent"	"Target"	"Spherical-dense"	"Limbus" and "crescent"		
A	mitochondria	—	24	64	12	48	38	14	48.6	20
		+	45	41	14	73	19	8	40.1	
B	mitochondria	—	0	58	42	57	33	10	83.7	30
		+	34	55	11	76	15	9	84.4	
C	cyclophorase	—	0	45	55	69	25	6	347.0	30
		+	6	72	22	77	8	15	302.0	
D	cyclophorase	—				32	51	17	1129.0	215
		+				64	22	14	956.0	

TABLE II a. Both the mitochondrial and cyclophorase preparations were made in 0.25 M sucrose. In the experiments using mitochondrial suspensions the components and manipulation of the Warburg flasks were as described in Table I, except that NaF was added only where indicated and glucose and hexokinase were omitted from all flasks. In the experiments where cyclophorase was used, 1.01 ml of the suspension was added to each flask. The flasks were made up to a final volume of 3 ml and contained per ml fluid the following additions: 1.5 μ moles of adenosine-5-phosphate; 17 μ moles of potassium phosphate buffer, pH 7.25; 10 μ moles of sodium α-ketoglutarate (added to the main chamber); 13 μ moles of $MgCl_2$; 11 μ moles of NaF in experiment C, and 16.5 μ moles of NaF in experiment D. The final sucrose concentration was adjusted to 0.25 M. The filter paper in the center well was saturated with 0.2 ml of 5 N NaOH and the manipulations were carried out as in Table I.

chondria are more proportional to the suppression of phosphorylation, the accompanying lowered rate of oxidation itself cannot be excluded as directly contributory to the mitochondrial changes.

In the experimental conditions established above to investigate the effect of osmolarity on phosphorylation, fluoride is used in order to block the action of adenosine-triphosphatase on the adenosine-triphosphate system. This conventional procedure is so arranged that the hydrolytic cleavage of ATP and the "leak" of high energy phosphate is almost eliminated and a high level of available ATP maintained (40). The use of fluoride has not prevented the degeneration of mitochondria due to variation in osmolarity (Table I). It remains to be seen if fluoride itself has an effect on structure. In Table II a it is seen that the addition of the fluoride exerts a decided action on the mito-

TABLE II b

The effect of fluoride on morphology and oxidation at various molarities of sucrose.

Experiment	Sucrose molarity	Fluoride	Morphology			μ liters O_2 uptake[1]
			"Target"	"Spherical-dense"	"Limbus" and "crescent"	
A	0.50	—	2	65	33	44.9
	0.50	+	46	40	14	37.5
	0.25	—	35	47	18	69.0
	0.25	+	62	27	11	46.5
B	0.50	—	3	68	29	34.5
	0.50	+	33	55	12	31.8
	0.25	—	40	45	15	59.7
	0.25	+	71	21	8	42.6
C	0.50	—	2	57	41	25.5
	0.50	+	16	67	17	26.0
	0.25	—	36	47	17	43.2
	0.25	+	42	40	18	26.2

TABLE II b. The mitochondria were prepared in 0.5 M sucrose. The components and manipulation of the Warburg flasks were as described in Table I, except that NaF was added only where indicated and glucose and hexokinase were omitted from all flasks. 20 mg of creatine were added to each flask in experiment B.

chondria form and sustains "targets" even though oxidation is often slightly depressed by the ion. The use of fluoride and of the proper osmolarity (Table I) which facilitates phosphorylation produces, therefore, a proportionally high concentration of "target" mitochondria. In addition, the independent action of fluoride in the stabilization of form is manifested in the "blanks" where α-ketoglutarate is omitted, oxidation is minimal and the "target" counts are unusually high. In these "blanks" where oxidation is minimal and adenylic acid is used as co-factor, the effect of fluoride alone is accentuated in preserving a high proportion of "target" mitochondria.

The relationship between the effects caused by variation in osmolarity and by addition of fluoride are clarified by further experiments. That degenerative effect of an increased osmolarity (e.g., 0.5 M sucrose) is partially counteracted by the fluoride is demonstrated in Table II b where, in the 0.5 M sucrose suspensions the fluoride maintains a concentration of "targets" equal to that found in 0.25 M sucrose suspension without fluoride. On the

[1] "Blanks" are not subtracted from the oxygen uptake values.

TABLE III

The effect of the molarity of sucrose and potassium chloride on ATPase activity of mitochondria.

Sucrose molarity	KCl molarity	μ moles inorganic P liberated
0.50	—	3.75
0.25	—	3.95
0.06	—	4.20
0.25	0.075	3.16
0.25	0.150	2.56
0.25	0.300	3.74

TABLE III. The reaction mixtures were made up in 20 ml beakers and had a final volume of 3.21 ml. Each ml of fluid contained the following additions: 11.7 μ moles of sodium diethyl barbiturate buffer, pH 7.4; 10.0 μ moles of $CaCl_2$; and 3.1 μ moles of potassium adenosine-triphosphate. Final sucrose and KCl concentrations were adjusted to the values indicated by adding appropriate quantities of sucrose and KCl solutions of suitable concentrations. After thermal equilibration, 0.4 ml of a mitochondrial suspension prepared in 0.5 M sucrose was added to each beaker.

contrary, it is also obvious from the data in Table II b that the omission of the fluoride enhances the effects of osmolar variation described in preceding experiments (Table I). This is evidence for the protective action of fluoride on mitochondrial morphology under conditions of osmolarity which uniformly eliminated the "target" or active forms. Both fluoride and isoosmolarity have common morphological effects.

Examination of these three tables (I, II a and II b) reveals that both high osmolarity and fluoride repress the rate of oxidation in mitochondrial suspensions. The phenomenon is not additive, however, because the addition of fluoride to a 0.5 M sucrose suspension does not augment the depression appreciably below what has already been incurred by the increase of osmolarity. It is pertinent to question from such evidence whether these agents act on the same phenomenon. Since the action of fluoride on mitochondrial ATPase is well established (27, 40), the effect of varied osmolarity on this enzyme was examined. It is seen in Table III that the variations of osmolarity with sucrose are slight and not comparable with the established action of fluoride, whereas the results obtained with K^+ ion are considerable and manifest irregular effects. The suppression of ATPase activity, the depression of oxidation and preservation of "targets" by the fluoride appear to be interlocked in a consecutive manner. The high sucrose osmolarity, which neither preserves form nor inhibits the ATPase appears to depress oxidation by a similar mechanism to that of fluoride, because the addition of fluoride

TABLE IV

The effect of the hexokinase trapping system on morphology.

Experiment	Hexokinase and glucose	Morphology			Oxidation and phosphorylation		
		"Target"	"Spherical-dense"	"Limbus" and "crescent"	μ liters O_2 uptake	μ moles inorganic P esterified	P/O
A	—	66	15	19	67.9	—	—
	+	0	64	36	69.6	11.8	1.90
B	—	77	19	4	68.1	—	—
	+	7	60	33	62.8	14.6	2.60
C	—	85	12	3	62.9	—	—
	+	13	73	14	58.8	12.2	2.32

TABLE IV. The mitochondria were prepared in 0.25 M sucrose. The components and manipulation of the Warburg flasks were as described in Table I. However, hexokinase and glucose were added to both phosphorylation and cytological flasks where indicated. The sucrose concentration in each flask was adjusted to 0.25 M.

in the presence of 0.5 M sucrose effectively protects the "target" mitochondria (presumably by ATPase inhibition) but is still without further influence on oxygen uptake. If an alternative mechanism were involved, the results on oxygen uptake might be an additive suppression. Since both fluoride and high osmolarity depress oxidation and have different effects on the ATPase and morphology, it is evident that the deleterious action of the 0.5 M sucrose can be related to unimpeded hydrolytic cleavage of ATP, which is not compensated by a suitable rate of oxidative phosphorylation. Although fluoride suppresses oxidation and the building up of ~P, it protects that reservoir by its specific ATPase inhibition with resultant maintenance of morphology.

It is observed, when the proper osmolarity is used and fluoride is added, that the employment of hexokinase as a trapping mechanism effectively and rapidly destroys the mitochondria. The high energy phosphate is removed from the medium and inaccessibly locked up in a low energy form as glucose-6-phosphate. This artificial leakage is associated with a degeneration of mitochondria, manifested by disappearance of "targets" and occurrence of numerous "crescents" (Table IV), whether AMP or ATP is used in the system (Table V). In this instance the result is the same as that occurring above either with a high osmolarity or with an ATPase activity uninhibited by fluoride. On the contrary, we observe that the creatine transphosphorylase system traps the high energy phosphate somewhat less effectively than does

TABLE V

Comparison of the effect of AMP and ATP with and without the hexokinase system on oxidation and morphology.

Experiment	Adenine nucleotide	Hexokinase and glucose	Fluoride	Morphology			μ liters O_2 uptake
				"Target"	"Spherical-dense"	"Limbus" and "crescent"	
A	AMP	—	—	71	19	10	79.2
	ATP	—	—	38	51	11	62.6
B	AMP	—	+	79	14	7	62.8
	ATP	—	+	67	16	17	43.1
C	AMP	—	+	85	11	4	56.8
	AMP	+	+	39	43	18	58.2
	ATP	—	+	57	29	14	41.6
	ATP	+	+	18	64	18	65.2
D	ATP	—	+	76	19	5	19.1
	ATP	+	+	29	58	13	54.0

TABLE V. The mitochondria were prepared in 0.25 M sucrose. The components and manipulation of the Warburg flasks were as in Table I, except that the NaF was added only where indicated, 3 μ moles of potassium adenosine-triphosphate per ml of fluid replaced the sodium adenosine-5-phosphate where indicated, and hexokinase and glucose were added to the side arms of the cytological flasks where indicated. The final sucrose concentration in each flask was adjusted to 0.25 M.

hexokinase, as was indicated by the lower average P/O ratios, ranging between 1.10 and 1.52. This less efficient trapping is attended by a slight or no alteration of the mitochondrial population. In our system hexokinase is an artificial trap which converts high energy phosphate into a loss because by our technique of preparation of the suspension the glycolytic enzymes are believed to be removed; but the creatine-phosphate trap accumulates as such in a reservoir which can be drawn back into the ATP system according to the level of the ATP present.

Another peculiarity of the hexokinase trapping system is that the rate of oxidation is unaltered or may be accelerated, depending on whether AMP or ATP is used, although the mitochondria still degenerate (Table V). This further supports the relationship between the destination of the ~P and the stabilization of structure; the failure to maintain the ~P is a prelude to mitochondrial degeneration, whether or not oxidation is disturbed or accelerated. The acceleration of oxidation with the hexokinase system is a noticeable and reproducible phenomenon that is observed when the system

TABLE VI

The effect of the creatine transphosphorylase system on oxygen uptake and phosphate distribution.

Experiment	Creatine	Adenine nucleotide	μ liters O_2 uptake	μ moles phospho-creatine	μ moles "labile ester"	μ moles "stable ester"	Total μ moles inorganic P esterified	P/O
A	—	AMP	44.8	0	3.6	0.8	4.4	1.05
	+	AMP	52.4	3.0	4.0	0	7.0	1.50
B	+	AMP	28.6				4.6	1.80
	+	ATP	18.1				3.8	2.34
	—	AMP	28.3[1]				—	—
	+	AMP	43.5[1]				—	—
	—	ATP	25.4[1]				—	—
	+	ATP	33.4[1]				—	—

TABLE VI. The mitochondria were prepared in 0.25 M sucrose. The components of the Warburg flasks were as in Table I, except that hexokinase and glucose were omitted. Instead, 20 mg of creatine were added, where indicated, to the main chamber of the flasks. 1.5 μ moles of ATP replaced the AMP where indicated. The final concentration of sucrose plus creatine in each flask was adjusted to 0.25 M.

is supplemented with ATP and is not seen when AMP is the co-factor used (Table IV). It is possible, on the basis of these experiments, to separate the effect of oxidation and phosphorylation on forms. The addition of ATP depresses oxidation slightly (Table V) but is able to maintain active "target" mitochondria, whereas the use of ATP together with hexokinase accelerates the rate of oxidation and is a demonstrably efficient phosphate trap, which destroys the mitochondria despite the increased respiration. The availability of ~P reservoir, not the rate of oxidation, is apparently decisive in the maintenance of the "target" mitochondria.

The ability of the hexokinase "trapping" system to accelerate oxidation in the presence of ATP and not with AMP is a peculiarity which has, under present conditions, little differential effect in relation to the alteration of the mitochondria. On the other hand, it is pertinent to the dynamics of the interrelation of oxidation and phosphorylation that an elevated oxidation rate is associated with an acceleration of phosphate transfer by means of hexokinase. The dampened oxidation due to either fluoride inclusion or ATP addition is relieved by draining ~P from the ATP and leaving the system ready for further phosphate acceptance, which seemingly augments oxida-

[1] "Blanks" are not subtracted from the oxygen uptake values.

TABLE VII

Effect of 2,4-dinitrophenol, Janus Green B and usnic acid on morphology, oxidation and phosphorylation.[1]

Experiment	Compound[2]	Morphology			Oxidation and phosphorylation			Per cent decrease due to compound	
		"Target"	"Spherical-dense"	"Limbus" and "crescent"	μ liters O_2 uptake	μ moles inorganic P esterified	P/O	"Target"	P/O
A	—	65	29	6	65.5	6.2	1.06		
	DNP	48	40	12	67.4	4.4	0.73	26	31
B	—	77	10	13	122.0	6.1	0.56		
	DNP	24	47	29	121.0	1.5	0.14	69	75
C	—	85	12	3	58.8	12.2	2.32		
	DNP	24	61	15	56.7	1.6	0.32	72	86
D	—	81	15	4	79.2	13.8	1.95		
	DNP	27	55	18	73.1	1.3	0.20	67	90
E	—	76	13	11	72.3	14.2	2.20		
	JG-B	52	26	22	58.1	7.1	1.37	32	38
F	—	71	25	4	46.8	12.0	2.29		
	JG-B	40	54	6	30.7	4.8	1.40	44	39
G	—	88	10	2	64.9	14.1	2.43		
	UA	39	50	11	63.5	5.1	0.90	56	63
H	—	85	9	6	57.0	14.3	2.81		
	UA	42	46	12	52.8	6.1	1.30	51	54

TABLE VII. The mitochondria were prepared in 0.25 M sucrose. The components and manipulation of the flasks were as in Table I except, where indicated, 20 mg of creatine were added to the main chamber of the flasks in place of hexokinase and glucose. Creatine was added to both phosphorylation and cytological flasks, whereas hexokinase and glucose were added only to phosphorylation flasks. Where indicated, the following additions were made per ml fluid: 12.3 γ of 2,4-dinitrophenol; 9.1 γ of Janus Green B; and 2.75 γ of usnic acid. The final concentration of sucrose or sucrose plus creatine in each flask was adjusted to 0.25 M.

tion (Table V). It is possible in our suspension of isolated mitochondria to speak of this deviation of the ~P away from its usual destination with hexokinase and ATPase as forms of "enzymatic uncoupling."

[1] In experiments A and B, creatine transphosphorylase is the trapping system; in all others, hexokinase is used.

[2] DNP = 2,4-dinitrophenol; JG-B = Janus Green B; UA = usnic acid.

The acceleration of oxidation is also observed in the use of the creatine transphosphorylation system, using either AMP or ATP as co-factors (Table VI). In these experiments ATP again manifests a dampening effect on oxidation as is observed in Table V. The morphology, although somewhat variable, is usually well sustained in these experiments as compared with the extreme degeneration uniformly encountered in the use of hexokinase. An analysis of a typical experiment for the distribution of ~P indicates that either as "labile phosphate" or as phosphocreatine there is a substantial quantity of ~P available. The fact that the creatine transphosphorylase system does not convert ~P to low energy phosphorus may account for the tendency of the creatine transphosphorylase mechanism to sustain morphology better than the hexokinase system does.

The mechanism of uncoupling oxidation and phosphorylation in relation to morphological alteration of mitochondria is further investigated by the use of 2,4-dinitrophenol, Janus Green and usnic acid. The 3 uncouplers used produce little or no change in oxidation rate, nearly obliterate the process of phosphorylation and lower the P/O ratio by a significant per cent (Table VII). The per cent decrease of "target" mitochondria is remarkably close in most instances to the amount by which the P/O is depressed. These experiments supplement the examples of "enzymatic uncoupling" by the hexokinase and ATPase and establish that the form of the mitochondria is dependent upon oxidation only to such extent as it furnishes ~P.

DISCUSSION

The efficiency of an enzyme system as an integrated complex depends not only on the adequate presence of the component catalysts but also on structural integrity, which assures both the stability in location of enzymes and their constant mutual accessibility. The necessity of structural relationships in the maintenance of optimal oxidative activity is emphasized by Keilin and Hartree (30) and by Slater (44) in their studies of the isolated succinoxidase system. Several other groups have been impressed by the structural complexity of such particulate systems, which lose their oxidative capacity when structure is disorganized. Ball and Cooper (1) and MacFarlane (35) found this true for succinoxidase activity in the isolated system and in the mitochondria, and independently concluded that the inhibition induced by their techniques was due to a disorientation of the enzymes, following disintegration of a lipoprotein complex, which cements together in close association groups of enzymes that perform together in cycles. This

agrees substantially with an earlier view of Stern (45), that such particles as he described were "subcellular" functional units composed of enzymes arranged so as to ensure a smooth functioning but highly complex mechanism for cellular oxidation. The work of Hogeboom and his colleagues (23) on cell fractionation led subsequently to the realization that the mitochondria were the morphological structures in which were situated the bulk of the enzymes comprising the Krebs citric acid cycle; and the mitochondria afforded a comprehensive unity to that system of oxidative enzymes. Schneider and Potter (43) refer to this functioning system as the "oxaloacetic acid oxidase" system and indicated its similarity to Green's "cyclophorase (15);" both complexes are associated with the mitochondria.

Further enlightening studies by Potter and his group (37, 41) provided much evidence for the great importance of the environment and the purity of particulate suspensions in the maintenance of integrated oxidative systems. Exploration of several factors showed that oxidations were sustained over long periods by mitochondrial suspensions, provided conditions were established to preserve the mechanisms of oxidative phosphorylation. The value of oxidation itself in the perpetuation of the oxidative system is limited by its ability to provide utilizable energy in the form of high energy phosphate. This link in the energy transfer system is most fragile and is easily uncoupled. Comparable studies of the relationship between oxidation and the internal structure of mitochondria by Harman and Feigelson (18) permitted the formulation of conditions which defined the interdependence of form and respiratory function. The present study emphasizes that the property of oxidative phosphorylation is the essential mechanism for maintenance of mitochondrial form and is analogous to the chemical observations of Pardee and Potter (37), although the precise conditions established by them were not studied. It is concluded, however, in view of the mutual fragility of oxidative phosphorylation and mitochondrial structure, that one of the primary effects of their carefully controlled environmental conditions is the preservation of mitochondrial integrity. A considerable fraction of the $\sim$P contributed by the processes of cellular oxidation is no doubt utilized in the maintenance of mitochondrial morphology of the *segregated* particles.

It is clear, therefore, that the *in vitro* osmotic environment is a determinant of the capacity of the mitochondrial systems studied to preserve a steady state (20), which is a fundamental characteristic of open biological systems (6). The oxidations are physiologically geared for the conversion of their energy through high energy phosphate bonds into synthetic mechanisms of several types, such as nucleoproteins (33), phospholipid (13), and phosphoproteins

(4), which preserve structural relationships and perpetuate the system itself. As Dixon (9) has pointed out, the remarkable result is the maintenance of a system of unstable biocatalysts by the occurrence of the reactions they catalyse. Interference with the integrated system permits disengagement of the co-enzymes (25, 43) and, if manipulation is severe, an irreparable impairment of the sustaining oxidative phosphorylation, which Lehninger (32) has shown to be co-enzyme linked. Our studies of the morphological effects of "enzymatic" and "chemical" uncouplers provides evidence of the participation of the phosphorylation in this autoperpetuation of the integrated oxidative system.

The pleomorphism of the mitochondria which we have observed *in vitro* and its relation to altered respiratory metabolism and phosphorylation suggests that form may exert an influence on cellular respiration *in vivo*. Many workers have examined the *in vivo* variations of mitochondrial shape and size under experimental conditions and propound the hypothesis that a change of mitochondrial form is a mechanism for the regulation of various cellular functions such as fluid balance (28), storage of glycogen (16) and protein metabolism (46). However, Roberts (42) has critically analyzed the existing evidence and on the basis of his further studies restricts himself to the conclusion that changes observed in the mitochondria are non-specific manifestations of an alteration in basic normal cellular metabolism. Valid proof of the exact role of the mitochondria in the regulation of cellular processes is still lacking. We believe it is also possible, contrary to such opinions, that many extreme variations of form are indications of functional derangement, represent responses to various stresses, and are not necessarily correlated with normal regulation of cell metabolism under physiological conditions. In fact, the configuration of the mitochondria is remarkably uniform under normal conditions, with certain minor variations. As suggested elsewhere (18), the stability of form may be associated with the dampened, controlled metabolism which characterizes the intact cells (3).

At present, our own observations conform more precisely with the hypothesis that the stabilization of the mitochondria is necessary for the normal balance of cellular respiration, which is affected by factors other than mitochondrial shape alone. The preservation of shape and normal function are mutually dependent and the intracellular environment is the protection providing a morphological state optimal for the requirements of cellular function. Wide fluctuations of form are pathological phenomena, as both Opie's (36) and Zollinger's (48) studies would also indicate, and are signs that mitochondria are not participating normally in the cellular metabolism.

SUMMARY

1. The osmolar concentration of the sucrose medium suspending isolated heart mitochondria influences the rate of oxidation and oxidative phosphorylation.

2. Low rates of oxidation and oxidative phosphorylation are associated with degenerative changes in the mitochondria.

3. Such degenerative changes persist when phosphorylation is uncoupled from oxidation, or the high energy phosphate bonds are filtered off irreversibly into an efficient trapping system.

4. Fluoride, which is an ATPase inhibitor, protects mitochondrial form.

5. The alterations in mitochondrial configuration are closely associated with metabolic changes in the mitochondrial suspensions, although they are not extrapolated back to the cell as controllers of cellular metabolism.

REFERENCES

1. Ball, E. C., and Cooper, O., *J. Biol. Chem.*, **180**, 113 (1949).
2. Barron, E. S. G., *Advances in Enzymol.*, **3**, 178 (1943).
3. Barron, E. S. G., Ardao, M. I., and Hearon, M., *J. Gen. Physiol.*, **34**, 211 (1950).
4. Barth, L. G., and Barth, L. J., *J. Exptl. Zool.*, **116**, 99 (1951).
5. Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29**, 379 (1946).
6. Bertalanffy, L. von, *Science*, **111**, 23 (1950).
7. Bodine, J. H., and Lu, K., *Physiol. Zoöl.*, **24**, 120 (1951).
8. Clowes, G. H. A., Keltch, A. K., Strittmatter, C. F., and Walters, C. P., *J. Gen. Physiol.*, **33**, 555 (1950).
9. Dixon, M., Multi-Enzyme Systems. Cambridge University Press, Cambridge, p. 13, 1949.
10. DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, **150**, 185 (1943).
11. Elliott, K. A. C., and Libet, B., *ibid*, **143**, 227 (1942).
12. Fiske, C. H., and Subbarow, Y., *ibid*, **81**, 629 (1929).
13. Friedkin, M., and Lehninger, A. L., *ibid*, **177**, 775 (1949).
14. Green, D. E., Mechanisms of Biological Oxidations. Cambridge University Press, Cambridge, p. 164, 1941.
15. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172**, 389 (1948).
16. Hall, E. M., and MacKay, E. M., *Amer. J. Path.*, **9**, 205 (1933).
17. Harman, J. W., *Exptl. Cell Res.*, **1**, 382 (1950).
18. Harman, J. W., and Feigelson, M., *ibid*, **3**, 47 (1952).
19. —— *ibid*, **3**, 58 (1952).
20. Hill, A. V., Adventures in Biophysics. University of Pennsylvania Press, Philadelphia, p. 55, 1931.
21. Hirshberg, E., and Rusch, H. P., *J. Cellular Comp. Physiol.*, **37**, 323 (1951).
22. Hogeboom, G. M., and Schneider, W. C., *Nature*, **166**, 302 (1950).
23. Hogeboom, G. M., Schneider, W. C., and Palade, G. E., *J. Biol. Chem.*, **172**, 619 (1948).
24. Hotchkiss, R. D., *Advances in Enzymol.*, **4**, 188 (1944).
25. Huennekens, F., and Green, D. E., *Arch. Biochem.*, **27**, 428 (1950).
26. Johnson, R. B., Feldott, G., and Lardy, H. A., *Arch. Biochem.*, **28**, 317 (1950).
27. Judah, J. D., and Williams-Ashman, H. G., *Biochem. J.*, **48**, 33 (1951).
28. Kater, J. M., *J. Morphol.*, **61**, 473 (1937).
29. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London*, **129**, 277 (1940).
30. —— *Biochem. J.*, **44**, 205 (1949).

31. Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, **179**, 957 (1949).
32. Lehninger, A. L., *ibid*, **190**, 345 (1951).
33. LePage, G. A., *Cancer Res.*, **9**, 297 (1949).
34. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162**, 421 (1946).
35. MacFarlane, M. G., *Biochem. J.*, **47**, xxix (1950).
36. Opie, E., *J. Exptl. Med.*, **86**, 45 (1947).
37. Pardee, A. B., and Potter, V. R., *J. Biol. Chem.*, **181**, 739 (1949).
38. Potter, V. R., *J. Cellular Comp. Physiol.*, **26**, 87 (1945).
39. —— *J. Biol. Chem.*, **169**, 17 (1947).
40. Potter, V. R., LePage, G. A., and Klug, H. L., *ibid*, **175**, 619 (1948).
41. Potter, V. R., Lyle, G. G., and Schneider, W. C., *ibid*, **190**, 293 (1951).
42. Roberts, H. S., *Anat. Rec.*, **104**, 163 (1949).
43. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **177**, 893 (1949).
44. Slater, E. C., *Biochem. J.*, **45**, 1 (1949).
45. Stern, K., *Cold Spring Harbor Symposia Quant Biol.*, **7**, 312 (1939).
46. Steffens, H. W., *Anat. Rec.*, **81**, 243 (1941).
47. Tyler, D. B., *Arch. Biochem.*, **25**, 221 (1950).
48. Zollinger, H., *Schweiz. Z. Path. u Bakt.*, **11**, 617 (1948).

THE EFFECTS OF SODIUM PARAMINOSALICYLATE ON THE GROWTH OF PURE CULTURES OF EMBRYO CHICK MYOBLASTS

G. ATTARDI, B. VITERBO, and D. GANDINI

Istituto di Istologia ed Embriologia Generale dell' Università di Padova, Italy

Received August 25, 1951

With respect to the chemical structure paraminosalicylic acid (P.A.S.) has certain similarities with paraminobenzoic acid (P.A.B.). The auxinosimilar action on seed germination and the stimulating influence on wound healing of the latter is known. These writers found it interesting to study the effects of the sodium salt of P.A.S. on embryonic chick tissues cultured *in vitro*, in order to establish the influence of this substance on animal cells in full proliferation. Reference may in this connection be made to the research work of Mascré and Deysson (15) carried out at the same time as the experiments to be reported here, but on other material. These authors studied the influence of P.A.S. as sodium salt on the division of plant cells. They observed that at a concentration of 0.25 per cent or more, P.A.S. Na has a lethal action on the cells of root meristems of *Allium cepa*. At a concentration of 0.010 per cent or less, P.A.S. Na has a mito-depressive action on these cells; at intermediate concentrations it blocks mitosis in the anaphase stage and causes typical changes of the chromosomes (shortening, conglutination).

MATERIAL AND METHODS

Apart from a few orientating studies on explants of chick embryo heart muscle, the experiments were carried out on pure cultures of dedifferentiated[1] myoblasts, obtained from pieces of ventricular myocardium of 8 days old chick embryos. At least six transfers were made every 48 hours.

For the quantitative estimation of the effects of P.A.S. Na Ebeling's method (5) for measuring the growth areas was employed. This method reflects what might be called the growth balance of a culture. This balance is the result of three positive

[1] We refer in this way to cells with uniform morphological characteristics that migrate in the myocardium cultures of young chick embryos, according to the opinion of some authoritative workers (8–12, 17–19). It is, however, noteworthy that other authors (1, 2, 3, 4–6, 7, 13, 14) maintain that after a certain number of transfers, the myoblasts disappear and pure cultures of fibroblasts and endothelial cells are obtained.

and three negative factors: cell division, migration toward the periphery and enlargement of the individual elements; cellular destruction, migration toward the explant and shrinking of the elements (16). These phenomena are strictly interdependent and as is specifically the case regarding cellular proliferation and migration, it is known that the factors affecting one also influence the other phenomenon, though not always proportionally. Even though Ebeling's method is not perfect, most of all because it does not take into consideration the density of growth of the cultures but only the superficial extension, it must be recognized that it is sufficiently accurate as compared with other biological methods of measurement. On the other hand, there should not be noticeable differences in density under optimal experimental conditions. Ebeling himself showed that the experimental error oscillates between 5 and 7 per cent, when particular care is taken in the choice of the cultures for experimentation, in the preparation of the nutritive medium, in subculturing, and above all in the final selection of the cultures to be examined.

The cultures were prepared on coverslips by the usual hanging drop method; the nutritive medium consisted of chick plasma, with the addition in equal parts of eight day old chick embryo extract. The plasma was diluted before use to half strength with different solutions of very pure sodium paraminosalicylate in Ringer solution; in the control cultures plasma diluted in the same proportions with Ringer solution was used, so that an equal dilution of the nutritive medium was obtained. Particular care was taken in the preparation of the P.A.S. Na solutions, since the substance is easily oxidized: the powdered product was used, already sterilely packed in vials in a nitrogenous atmosphere. The solutions were prepared with sterile Ringer solution immediately prior to their use. In this way we tried to reduce to a minimum the loss of substance caused by the preliminary manipulations. The final concentrations of P.A.S. Na in the nutritive medium were 2, 5, 10, 25, 50, 100, 300, 500 and 1 000 mg per 100 cc.

Particular care was taken when chosing cultures for the experiments, so that only those well grown in the previous transfers that showed a large growth area and good cellular density were used. Each fragment was as far as possible divided into two equal parts, one of which was used for the experimental culture and the other for the control. We tried to place the twin fragments in the same conditions, transferring them into drops of nutritive medium of the same size and using glasses with concavities of equal volume. Immediately after the preparation the outlines of the cultures were drawn by means of a projection apparatus, proceeding as quickly as possible in order to avoid overheating. After 24 hours the cultures were examined under the microscope and all those contaminated with germs or with molds were discarded. Only cultures of regular growth were used for graphic representation (by means of the projector with the same magnification). The migration zone (fig. 1) in these cultures corresponded to the "reticular radiating outgrowth" of W. Lewis and to the "geordnetes Wachstum" of P. Weiss. At 48 hours the outline was redrawn. Some cultures in which growth continued abundantly and densely were watched until the 72nd hour and further until the 96th hour (fig. 2). When drawing the graphs, elements or groups of elements that were isolated in the periphery of the growth zone were excluded from the outline.

The areas outlined on the graph were successively measured with an Amsler polar planimeter; the total area of the culture at a certain time of growth minus the area

Fig. 1. Culture of myocardium of 8 day old chicken embryo at the 7th transfer (fixed after 48 hours of life "*in vitro*" and stained with Harris' hematoxilin). Microphot. 82 ×.

of the original fragment expresses the "absolute growth" of the fragment itself; the ratio between it and the original surface represents the "relative growth." The ratio between the relative growth of the experimental culture and that of the control culture constitutes the "growth index." When designating the initial area of the experimental culture and the control by A and A′ respectively, and the total area of the same cultures after a certain period of life *in vitro* by B and B′, we first get a relative growth:

$$E = \frac{B - A}{A}$$

and in the second place:

$$C = \frac{B' - A'}{A'}$$

The growth index will then be:

$$I = \frac{E}{C}$$

All cultures were fixed in Helly's (Zenker-formol) and stained with Harris's hematoxilin to allow a qualitative study of the cellular elements.

Fig. 2.

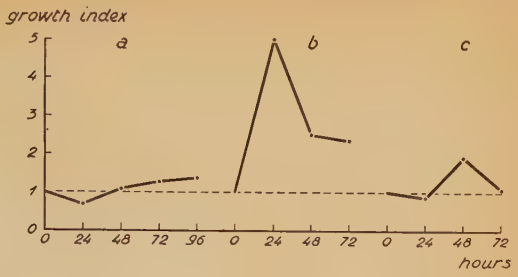


Fig. 3.

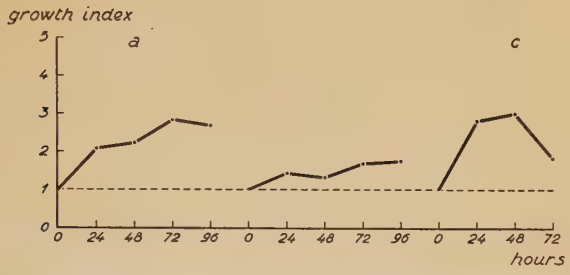


Fig. 4.

Fig. 2. Graph which shows the outlines of an experimental culture (a) and the relative control (b), respectively at the start and after 24, 48 and 72 hours of life *"in vitro"*.

Fig. 3. Graphs which show the behavior, in function of time, of growth index of the cultures 348 (a), 358 (b), 349 (c).

Fig. 4. Graphs which show the behavior, in function of time, of growth index of the cultures 335 (a), 354 (b), 351 (c).

RESULTS

Sodium paraminosalicylate in concentration of 2 mg per 100 cc had only a slight growth-promoting effect on the cultures of myoblasts (Table I).

The concentrations of P.A.S. Na between 5 and 100 mg per 100 cc. revealed on the other hand a clear stimulating action (Table II).

TABLE I

Addition to nutritive medium	No. culture		24 hrs.	48 hrs.
P.A.S. Na 2 mg per 100 cc Ringer	327	R.G.	3.33	18.07
	327 a	R.G.	2.93	17.43
		I.	1.14	1.04
P.A.S. Na 2 mg per 100 cc Ringer	328	R.G.	7.38	23.25
	328 a	R.G.	6.00	20.64
		I.	1.23	1.13

(R. G. = relative growth; I = growth index).

Table II

Addition to nutritive medium	No. culture		24 hrs.	48 hrs.	72 hrs.	96 hrs.
P.A.S. Na 5 mg per 100 cc Ringer	333	R.G.	6.40	27.00	—	—
	333 a	R.G.	10.00	25.50	—	—
		I.	0.64	1.06	—	—
P.A.S. Na 5 mg per 100 cc Ringer	335	R.G.	6.50	23.75	36.50	43.00
	335 a	R.G.	3.12	10.62	12.87	16.00
		I.	2.08	2.23	2.83	2.69
P.A.S. Na 10 mg per 100 cc Ringer	338	R.G.	7.59	21.24	—	—
	338 a	R.G.	3.82	13.45	—	—
		I.	1.99	1.58	—	—
P.A.S. Na 10 mg per 100 cc Ringer	339	R.G.	9.27	31.80	—	—
	339 a	R.G.	4.46	16.00	—	—
		I.	2.08	1.99	—	—
P.A.S. Na 25 mg per 100 cc Ringer	345	R.G.	5.00	15.66	—	—
	345 a	R.G.	3.09	13.18	—	—
		I.	1.72	1.19	—	—
P.A.S. Na 25 mg per 100 cc Ringer	348	R.G.	3.16	9.32	19.42	24.47
	348 a	R.G.	4.73	8.64	15.36	17.90
		I.	0.68	1.08	1.26	1.37
P.A.S. Na 25 mg per 100 cc Ringer	349	R.G.	2.00	6.90	12.60	—
	349 a	R.G.	2.29	3.57	11.86	—
		I.	0.87	1.93	1.06	—
P.A.S. Na 50 mg per 100 cc Ringer	351	R.G.	5.00	11.66	18.11	—
	351 a	R.G.	1.78	3.89	9.89	—
		I.	2.81	2.99	1.83	—
P.A.S. Na 50 mg per 100 cc Ringer	352	R.G.	4.92	17.33	—	—
	352 a	R.G.	3.33	13.00	—	—
		I.	1.48	1.33	—	—
P.A.S. Na 50 mg per 100 cc Ringer	354	R.G.	3.73	10.07	17.13	21.53
	354 a	R.G.	2.61	7.67	10.22	12.33
		I.	1.43	1.31	1.68	1.75
P.A.S. Na 100 mg per 100 cc Ringer	358	R.G.	7.00	15.29	25.29	—
	358 a	R.G.	1.40	6.07	10.73	—
		I.	5.00	2.52	2.36	—
P.A.S. Na 100 mg per 100 cc Ringer	359	R.G.	7.00	16.87	—	—
	359 a	R.G.	4.58	14.37	—	—
		I.	1.53	1.17	—	—

TABLE III

Addition to nutritive medium	No. culture		24 hrs.	48 hrs.	72 hrs.	96 hrs.
P.A.S. Na 300 mg per 100 cc Ringer	362	R.G.	4.22	11.00	19.22	29.44
	362 a	R.G.	11.50	21.33	34.00	45.00
		I.	0.36	0.52	0.58	0.65
P.A.S. Na 500 mg per 100 cc Ringer	365	R.G.	1.44	8.00	—	—
	365 a	R.G.	2.94	11.67	—	—
		I.	0.49	0.78	—	—
P.A.S. Na 500 mg per 100 cc Ringer	366	R.G.	7.60	12.40	—	—
	366 a	R.G.	7.62	17.25	—	—
		I.	0.99	0.72	—	—
P.A.S. Na 1 000 mg per 100 cc Ringer	370	R.G.	0.50	3.50	—	—
	370 a	R.G.	5.29	13.14	—	—
		I.	0.09	0.27	—	—
P.A.S. Na 1 000 mg per 100 cc Ringer	371	R.G.	2.00	3.51	—	—
	371 a	R.G.	5.09	13.00	—	—
		I.	0.39	0.27	—	—

Table II indicates that, in relation to the concentrations of P.A.S. Na between 5 and 100 mg, the growth index values are mostly greater than unity, even in the range of noticeable variations: the latter can be related either to an individual difference of response of the single cultures, or to the interference of extrinsic factors difficult to control. The variability in behaviour of the single cultures also manifests itself as regards the behavior of growth stimulation in function of time. Thus in some cultures (nos. 333, 348) the growth index presents values lower than unity after 24 hours of life *in vitro*, with a clear tendency, however, to increase during the following days, so that after 48 hours the relative growth of the experimental cultures slightly surpasses that of the controls; the stimulating action becomes evident in the third and fourth day in culture no. 348 (fig. 3 a). Other cultures (nos. 338, 345, 358 (fig. 3 b), 359) show a maximum of stimulating action the first day with a rapid decrease the next day, nevertheless remaining within the range of values greater than unity.

Characteristic is the behavior of culture no. 349 (fig. 3 c), for which the growth index gives a value slightly lower than unity at the 24th hour, rises sharply to the value of about 2 after 48 hours, and the third day declines rather rapidly to 1. Culture no. 351 also presents the maximum stimulating action at the 48th hour, but unlike culture no. 349, this action is al-

ready noticeable the first day (fig. 4 c). In other cultures finally (nos. 335, 354 (figs. 4 a, b)) the maximum value of growth index is reached from the third to fourth day.

The concentrations from 300 up to 1 000 mg per 100 cc show a definite inhibiting action on growth (Table III). One must point out, however, that fluctuations of intensity are observed here too from case to case, although of lesser degree than in the lower concentrations.

The examination of the fixed and stained cultures did not reveal substantial differences between experimental and control cultures with regard to the character of growth and the general morphological physiognomy of the elements. Simultaneously there was no evidence of regressive changes in the cytoplasm or nucleus that could be referred to the action of P.A.S. Na. Only in cultures treated with the greatest concentration of 1 g per 100 cc, slight vacuolization was observed. This was, on the other hand, absent in the controls. The vacuoles consisted of rather big drops of fat present in most elements in the periphery of the migration zone, which otherwise presented no signs of cytoplasmic or nuclear damage. Nevertheless, even though these phenomena of vacuolization must depend on the presence of strong doses of P.A.S. Na, they were not important enough to explain the low growth rate of the relative cultures.

DISCUSSION AND CONCLUSIONS

The investigations reported in this paper have demonstrated a clear stimulating action on the growth of pure cultures of chick embryo myoblasts by P.A.S. Na, added to the nutritive medium in concentrations ranging from 5 to 100 mg per 100 cc. As already recorded, Mascré and Deysson (15) showed in plant material a blocking effect of the same concentrations of P.A.S.Na on mitosis, accompanied by typical changes of the chromosomes.

In our experiments, however, many kariokineses, perfectly normal in all their phases, were present in the treated cultures. For concentrations higher than 300 mg per 100 cc, we did not find the lethal effect on the cells as described by the French authors for the dose of 250 mg per 100 cc. Only a slowing down of growth was observed which can be attributed to a specific action of the higher concentrations of the substance. For the largest dose used (1 g per 100 cc), however, a certain influence of the increased osmotic pressure of the medium cannot be excluded (equal to that of a 12.71 per cent NaCl solution).

The disagreement between our results and those of the French authors

could also, apart from the difference in material, depend upon the different experimental conditions of life the tissues were exposed to. It may also be said that, in contrast to the results obtained by Mascré and Deysson, ours adduce evidence in favour of a parallelism in activity between P.A.S. and P.A.B., a parallelism that must be due to the similarity in the chemical structure of the two substances.

It must, on the other hand, be stated that there is a certain agreement between our experimental results and those furnished by the clinical observations with regard to the improvement of the blood crasis and general trophism. This is often found in patients treated with P.A.S. Na, independently of and contrary to the anatomic-pathological evolution of the process, when blood concentrations of the drug, equivalent to those employed in the experiments described above, were used.

SUMMARY

1. The action of sodium paraminosalicylate (P.A.S. Na) was studied upon the growth of pure cultures of dedifferentiated myoblasts, obtained from pieces of ventricular myocardium of eight day old chick embryos by means of repeated transfers. For measuring the rate of growth *in vitro* Ebeling's method was applied. The concentrations of substance used, expressed in mg per 100 cc of nutritive medium (plasma + Ringer + embryonic extract) were 2, 5, 10, 25, 50, 100, 300, 500 and 1 000.

2. The concentration of 2 mg per 100 cc of P.A.S.Na has shown only a slight stimulating action on cellular growth.

3. The concentrations between 5 and 100 mg per 100 cc have revealed a marked stimulating activity upon growth.

4. The concentrations between 300 and 1 000 mg per 100 cc have instead shown a definite inhibiting effect on growth *in vitro* of the myoblasts.

5. The examination of the fixed and stained cultures has not shown regressive changes of the elements in the migration zone, with the exception of cultures treated with the highest concentration (1 gr per 100 cc), in which slight vacuolization was observed.

REFERENCES

1. Burrows, M. T., *J. Exptl. Zool.*, **10**, 63 (1911).
2. Carrel, A., *Berlin. Klin. Wochschr.*, **51**, 509 (1914).
3. —— *J. Exptl. Med.*, **20**, 1 (1914).
4. Ebeling, A. H., *ibid*, **30**, 531 (1919).
5. —— *ibid*, **34**, 231 (1921).
6. —— *ibid*, **35**, 755 (1922).

7. Fischer, A., Gewebezüchtung. R. Müller und Steinicke, München, 1927.
8. Levi, G., *Arch. Sci. Biol.* (*Italy*), **40**, 1 (1916).
9. —— *Arch. Ital. Anat.*, **16**, 423 (1919).
10. —— *Accad. Naz. Lincei, Roma*, **32**, 59 (1923).
11. —— *Compt. Rend. Assoc. Anat., 19ème Réun.*, p. 204 (1924).
12. —— *Ergeb. Anat. Entwicklungsgeschichte*, **31**, 125 (1934).
13. Lewis, W., *Anat. Rec.*, **25**, 111 (1923).
14. —— *Carnegie Inst. Wash. Publ.*, **363**, 1 (1926).
15. Mascré, M., and Deysson, G., *Ann. Pharm. Franç.*, **8**, 604 (1950).
16. Mayer, E., *Arch. Exptl. Zellforsch. Gewebezücht.*, **10**, 221 (1931).
17. Olivo, O., *Mon. Zool. Ital.*, **36**, 171 (1925).
18. —— *Compt. Rend. Assoc. Anat., 21ème Réun.*, p. 445 (1926).
19. —— *Arch. Exptl. Zellforsch. Gewebezücht.*, **8**, 250 (1929).

CELL SUSPENSIONS FROM ORGAN RUDIMENTS OF CHICK EMBRYOS

ARON MOSCONA[1]

Strangeways Research Laboratory, Cambridge, England

Received September 8, 1951

In this communication a method is described whereby the limb buds and the mesonephros of early chick embryos have been broken down into suspensions of discrete, viable cells. The method was developed in the course of a study on the differentiation of embryonic tissues following partial and complete disruption of their histological pattern. The experiments with cell suspensions were undertaken with the aim of studying the capacity of the discrete cells to reaggregate and the extent to which such aggregates will differentiate and develop.

The usual techniques for obtaining tissue suspensions are based on mechanical disintegration of the tissues. Such methods, being applicable only when a relatively large quantity of tissue is available, are not suitable for the disintegration of early embryonic organ rudiments. Moreover, a process was required, whereby the disintegration would proceed gradually and so far as possible under controllable conditions. It is well known that certain proteolytic and hydrolytic enzymes dissolve intercellular ground substances (2, 7, 11), and an attempt was therefore made to release the cells into suspension by using such enzymes to break down the intercellular matrix of the rudiments.

TECHNIQUE

Experiments were carried out with hyaluronidase[2] and with trypsin. Solutions of hyaluronidase preparations, when in contact with the tissue for a sufficiently long time to make the intercellular materials soluble, were observed to cause irreversible abnormalities in a high percentage of the cells. These might have been due to the action of the enzyme, to the presence of some impurity in the preparation or to the prolonged exposure of the tissue to the solution. Proteolytic enzymes are known to

[1] The Hebrew University, Jerusalem. This work has been made possible by a fellowship granted by the Humanitarian Trust, London.

[2] Armour bovine hyaluronidase, obtained through the courtesy of Dr. L. L. Lachat and Benger's 'Hyalase' were used.

dissolve the ground substanes of certain tissues more rapidly than hyaluronidases (3, 7), and it has been demonstrated repeatedly that trypsin, even in relatively high concentrations, does not attack living cells (9, 10, 13, 14). Extensive tests have convinced us that the cells of the limb bud and the mesonephros are very resistant to the action of trypsin, and when cultivated in tissue culture or as chorioallantoic grafts after treatment with this enzyme, they pursued their characteristic course of development. The method was as follows.

The limb buds were dissected aseptically from 3- or 4-day embryos and transferred to a hollow-ground slide, in which the rest of the treatment was performed under microscopic control. The dorsal and ventral epidermal ridges were cut off and the buds rinsed repeatedly in Tyrode solution. They were then transferred to a saline lacking calcium and magnesium salts (NaCl 8.00, KCl 0.20, $NAH_2PO_4.H_2O$ 0.00.5, $NaHCO_3$ 1.00, glucose 2.00–gm per 11.H_2O) in which they were incubated for 15 min. at 38° C. In this saline the tendency of the tissue to stick to the glass was greatly increased.

After additional rinsing, the limb buds were transferred to a 3 per cent solution of B.D.H. Trypsin in the above saline which was sterilized by Seitz-filtration. After 10 to 12 min. at 38° C, the adhesiveness of the cells both to the glass and to each other decreased considerably. The epidermis became completely detached from the underlying tissue and could be easily shaken off the limb bud. A boundary between the outer, myogenic, and the inner chondrogenic (5) blastema became distinct and they could be fairly cleanly separated from each other. Each part was cut into smaller pieces which were transferred into fresh trypsin medium, the pH of which was gradually raised to 8.4–8.6 by the addition of sterile 1 per cent KOH solution.

After 15 to 20 min. in this medium at 38° C the cells became loosened and many of them were to be found free in the fluid. The remaining pieces could be very easily broken up into discrete cells in suspension by sucking them once or twice through a pipette of a suitable bore (figs. 1, 2). The suspended cells were washed twice by centrifugation in the Ca and Mg free saline and in Tyrode, then resuspended for cultivation in the fluid exudate from a plasma-embryo extract clot. The exudate was prepared (according to a method devised by H. B. Fell—personal communication) by mixing fowl plasma and extract from an 11-day embryo in equal quantities; the ensuing clot was crushed and incubated at 38° C for one hour; the collected exudate was centrifuged at 2 500 r.p.m. for 15 min. to remove extraneous cells.

The mesonephroi from 3-day embryos were treated in essentially the same way. They were stripped of adherent mesenchyme and treated with the Ca and Mg free saline for 20 to 30 min. at 38° C. After thorough rinsing the tissue was transferred to the trypsin solution, cut into small fragments and incubated for about 20 min. Washing by centrifugation was preferably done in the Ca and Mg free saline only, as the presence of these ions increased the tendency of the cells to clump.

It will be seen that the method is based mainly on the following considerations. It has been shown (1, 8, 12) that the stability of the intercellular materials and the mutual adhesiveness of cells decrease through loss of calcium and magnesium, as when the tissue is bathed in a fluid free of these ions. It is also known (3, 8) that solutions with alkaline pH promote, under certain conditions, hydration and swelling of some intercellular substances. Neither of these factors alone will reduce the intercellular matrix of the rudiments studied to completely soluble compounds.

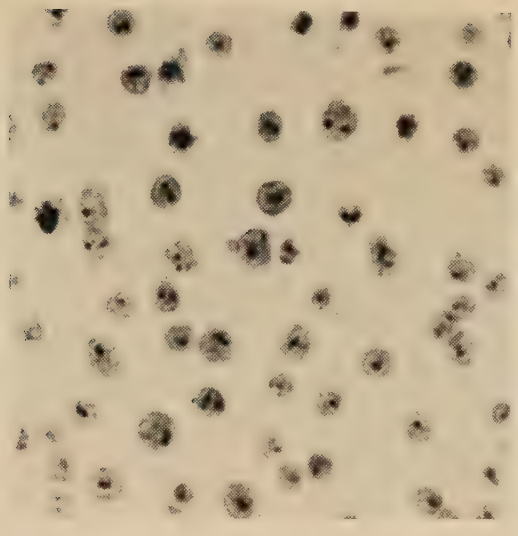


Fig. 1.

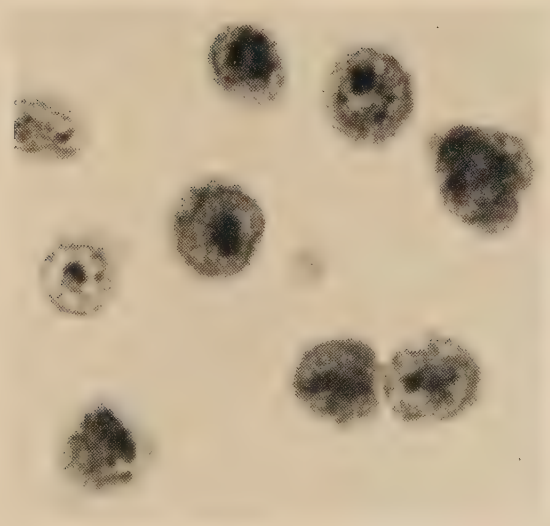


Fig. 2.

Fig. 1. Smear of a suspension of 4-day limb-bud cells, immediately after disintegration of the tissue in the trypsin medium. Formol vapour; Ehrlich's haematoxylin. ×240.
Fig. 2. Same preparation as in fig. 1, magnified ×1140. In the left bottom corner a cell in metaphase.

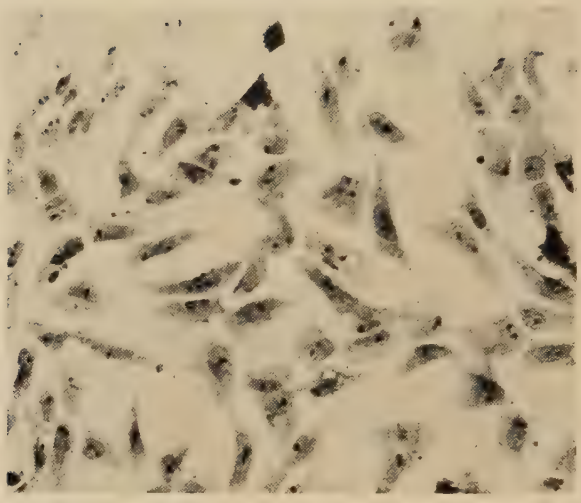


Fig. 3. Hanging drop culture of same cell suspension, 24 hours after explantation. 10 per cent formol-Tyrode; Mayer's haemalum. ×240.

However, their action, if applied in the sequence described above, renders the matrix liable to a quick digestion by trypsin, so that the cells can then be freed by rinsing.

The method is restricted in its application to early embryonic tissues. Its limitations are imposed by the type of the tissue and by the nature and composition of the intercellular materials.

OBSERVATIONS

The discrete cells. When observed in a fluid culture medium at 38° C[1] the discrete cells were seen to be moving actively on the floor of the glass vessel. Many of them showed variously shaped protrusions, some of which appeared to be pseudopodial in function, while others were evidently due to changes in the cell membrane or in the cytoplasm induced by the treatment. These changes were found to be reversible, provided that the nucleus was not affected. Myogenic cells proved to be the most sensitive to the conditions of the treatment and showed the highest percentage of nuclei undergoing karyolysis.

After about 30 min. in the fluid culture medium most of the cells assumed a rounded shape moving about with blunt 'lobopodia.' Movement was less active in a drop of cell suspension spread on a hanging drop clot; limb bud

[1] The constant temperature microscope stage used was devised by Dr. J. C. Heath from the S. R. L. to whom I am also indebted for advice in its use.

cells, for instance, soon became attached to the clot and most of them spread out into a fibroblastic form (fig. 3). Mitotic activity was resumed within 12 hours.

In hollow-ground slide cultures of cell suspensions grown in a liquid medium with a cell density of about 25×10^4 cells per 0.2 cc medium, most of the cells reaggregated within 24 hours into small clumps. These, when cultivated further by the watch glass technique (4), continued to differentiate attaining a remarkable degree of histological regulation. In mixed suspensions composed of cells from two types of tissue, the cells segregated during the formation of the clusters, the cells of each type forming a different part of the same cluster. These observations will be reported in detail later.

I wish to express my gratitude to Dr. H. B. Fell, Director of the Strangeways Research Laboratory, for her continuous interest in this study and for most valuable advice and criticism.

REFERENCES

1. Chambers, R., *Cold Spring Harbor Symposia Quant. Biol.*, **8**, 144 (1940).
2. Day, T. D., *Lancet*, **2**, 945 (1947).
3. —— *J. Physiol.*, **109**, 380 (1949).
4. Fell, H. B., *J. Roy. Microscop. Soc.*, **60**, 95 (1940).
5. Fell, H. B., and Robison, R., *Biochem. J.*, **23**, 767 (1929).
6. Gersh, I., and Catchpole, H. R., *Amer. J. Anat.*, **85**, 457 (1949).
7. Holtfreter, J., *Ann. N. Y. Acad. Sci.*, **49**, 709 (1948).
8. Jensen, A. A. Bohus, *Nature*, **162**, 931 (1948).
9. Medawar, P. B., *Nature*, **148**, 783 (1941).
10. Meyer, K., and Rapport, M. M., *Science*, **113**, 596 (1951).
11. Monné, L., *Experientia*, **2**, 1 (1946).
12. Moore, A. R., *Exptl. Cell Res.*, **2**, 284 (1951).
13. Northrop, J. H., *J. Gen. Physiol.*, **39**, 375 (1947).
14. Runnström, J., Monné, L., and Broman, L., *Arkiv Zool.*, **35 A**, No. 3 (1944).

ON THE IDENTITY OF "ISOLATED CHROMOSOMES"

I. COMPARISON OF CHROMATIN THREADS OBTAINED BY TWO RADICALLY DIFFERENT MECHANICAL MEANS[1]

A. R. T. DENUES

Sloan-Kettering Institute for Cancer Research, New York, N. Y.

Received September 16, 1951

The chromatin threads first isolated by Claude and Potter (2, 3) and by Mirsky and Pollister (9, 10) were thought by these workers to be morphologically related to the chromosomes of the resting nucleus, if not identical with them. Such threads were later definitely characterized by Mirsky and Ris (11) as "isolated chromosomes." Evidence for this was recently extended (12). However, objections to this characterization have appeared. Lamb (5, 6) concluded from an extensive study that such threads were not chromosomes but complex fragments of drawn-out nuclei; and Calvet, Siegel, and Stern (1) also concluded, in a preliminary note, that these bodies were artificial products derived from the "chromatin network." A summary of the evidence for the characterization as "isolated chromosomes" and a discussion of Lamb's conclusion were recently given by Ris and Mirsky (14). These and other contributions, to be critically discussed in a subsequent paper, indicate the desirability of further examination of this important question.

As a first step in a series of experiments, comparisons have been made of the threads obtained by two radically different mechanical means, *viz.*, the Waring Blendor and the Logeman Hand Colloid Mill. Such evidence will help clarify the question of identity, that cogent comparisons may then be made between normal and neoplastic materials.

MATERIALS AND PROCEDURE

The preparations were made after the general procedures of Claude and Potter (3) and of Mirsky, Pollister, and Ris (10, 11), consisting essentially of physical reduction of the material followed by differential centrifugation to obtain chromatin threads. The work was done in a cold laboratory and with an International Refrigerated Centrifuge PR 1 at temperatures in the range 0–5 degrees Centigrade.

[1] This investigation was made under a grant from the Alfred P. Sloan Foundation and was supported by grants from the National Cancer Institute, of the National Institutes of Health, Public Health Service, and from the American Cancer Society.

Blood of domestic chickens (Plymouth Rock) was used as the convenient source of nucleated erythrocytes. It was collected directly from severed carotid arteries in chilled 0.14 M NaCl containing neutral potassium oxalate (*ca.* 2 mg per anticipated ml of blood). Erythrocytes were separated by centrifugation at 600 g. The supernatant and buffy coat were decanted and the cells were washed *ca.* 4 times with saline. Physical reductions were effected either in a Waring Blendor (here abbreviated as *WB*) or in a Logeman Hand Colloid Mill (here abbreviated as *LM*) (4). Two separate runs were made with the Waring Blendor, three with the Logeman Mill.

The Blendor was used with the standard ½-liter screw-top fluted container charged with *ca.* 60 ml of a 20:1 dilution of the cleaned red cells in saline. Operation was at the full speed, found to be *ca.* 15 000 rpm, for five periods of one minute duration each, separated by cooling intervals. These conditions combined great turbulence with the high-velocity action of the impeller.

The Logeman Mill is a simple stainless steel extrusion device that applies relatively mild, pure shear to fluids forced by a hand-operated pump through the seat of a spring-loaded valve. The Mill, of ½-liter capacity, was modified by replacing the standard spring (7/8″—No. 18 AWG—8 turns/in.) with a similar spring one inch long made of No. 15 AWG steel wire (0.057″ diameter); this was used with or without 1/8″ additional compression by an insert. A charge of 100 ml of a 100:1 dilution of the cleaned red cells in saline was used. This material was recycled for ten extrusions.

The resulting mixes were separated by differential centrifugation at *ca.* 600 g, the sediment of threads being repeatedly washed with saline. Wet mounts were made for the dark contrast-medium oil immersion phase microscopy (970×) of blood, clean cells, and of material after each minute of treatment by Blendor and after each extrusion in the Logeman Mill. Systematic counts were made in random fields with the aid of a Howard micrometer disc, as reported previously (4). The resulting numbers of intact cells *C*, of free nuclei *N*, and of free threads *T* were combined into two convenient guiding parameters N/C and T/N. Permanent slides were also made of these materials, using Wright's, acetocarmine, or Feulgen stains.

Mounts for electron microscopy were made of unfixed material or of material fixed with acetic alcohol (1:3), acetocarmine, 2 per cent aqueous chromic acid or 2 per cent osmium tetroxide in water or in saline. The fixatives were generally mixed with bulk suspensions of the material, in excess; vapor-fixation with osmium tetroxide was also used. Suspensions of threads were dropped on collodion films and allowed to settle, the excess was removed, and the grids were washed ten times with glass-distilled water. In one run the supernatant from the first centrifugation was similarly mounted. Shadowing was with chromium at *ca.* 11 degrees.

In an effort to develop details of fine structure in one run, the threads were treated before regular fixation with boiling water or with M/16 Sørensen phosphate buffer at pH 8.

An RCA type EMU-2B electron microscope fitted with a bias gun and with an objective aperture 0.001 in diameter was used. At least two mounts of each preparation were scanned systematically, recording representative pictures. A total of *ca.* 1 000 individual micrographs was made, exclusive of controls. The analyses, comprising studies of general morphology and detailed measurements, were made from all the assembled micrographs with the help of systematic tabulations; illustrations were selected from these tabulations.

RESULTS

Uniform preparations of clean red cells of normal appearance were obtained as starting material in all runs. The studies of the disruption of the material indicated that the ratios N/C and T/N increased rather regularly and successively with treatment in all instances. With the Waring Blendor, N/C became infinite (i.e., the preparations were found microscopically acellular) after three minutes of treatment, and terminal values of T/N were of the order of 50. With the Logeman Mill, cells were readily destroyed and superior disruption of the nuclei was indicated. In a run without spacer, the terminal T/N was recorded as 29 but uncountable tangles of threads were present. In runs with the spacer, T/N was over 100 after but 4 extrusions in one, and the product in the other was found microscopically anuclear.

Stainings indicated that the threads from either the Waring Blendor or the Logeman Mill were positive for Wright's nuclear stain, for acetocarmine, and for the Feulgen reagent.

Light- and electron-micrographs disclosed a wide range of relatively straight and curved filaments in both kinds of material. The curved forms included wavy threads, V's, crescents, and J's. Figs. 1 to 4 are light-micrographs illustrating this common range of forms. Some recurrences of a given thread were strongly suggested in such plates. Some detailed structural features were also evident in the light microscope, including constrictions, bifurcated ends, and coiled aspect. As the material was of a size near the limit of resolution of this instrument, most of the work was done with the electron microscope. A few of the electron-micrographs illustrate for unpretreated material the range of forms mentioned above (Figs. 5–16):

	Waring Blendor	*Logeman Mill*
Relatively straight	Figs. 5, 6	Fig. 7
Wavy	8	9
V	5, 10	11
Crescent	5, 12	13
J	14	15, 16

Other examples of these forms include the wavy threads of Figs. 19, 20, 22, and 31; the V's of Figs. 30, 32, 37, and 38; and the crescents of Figs. 35 and 36.

In addition, special structural features were seen: The ends of these threads were generally smooth in appearance (*e.g.*, Figs. 17 and 18). They were also found to be tapered or ragged (Figs. 19 and 20) or, occasionally, to carry apparent "tails" (Figs. 17, 21, 22, and 23). Split ends (Figs. 24, 25, 26, 27) suggested double structure. This was seen in the body of some of the threads as well (Figs. 26, 27, 23). Lateral indications of structure were often seen (*e.g.*, Figs. 28, 29, 33), and lateral attachments or associated materials were sometimes encountered (*e.g.*, Figs. 8, 12, 18, 37, and 38). The regularity of some of the lateral structure suggested periodic longitudinal structure. Constrictions were commonly evident (*e.g.*, Figs. 30, 31, 32, 33, and 34, the last showing both "primary" and "secondary" constrictions); also see Figs. 7, 9, 10, 11, 14, 15, and 16. Recurrences were suggested, as for the J's in Figs. 15 and 16; for the crescent-shaped threads in Figs. 12, 35, and 36; and for the threads of Figs. 37 and 38 which show a marked crook near one end. Longitudinal variation in density was difficult to see here, the threads generally appearing opaque. It was often clear in lighter prints, however, and is suggested here in Figs. 5, 9, 10, 11, and 26. In the last, the variations in density appear in register for the two constituent threads. Most of the few persisting nuclei seen were round, but some apparently were extruding material. This was observed both in the processed material (Figs. 39 and 40) and in preparations of washed unfixed cells (Fig. 41). The supernatant examined in a run with the Logeman Mill was characterized by dense particulate matter ranging from 0.1 to 0.3 micron in diameter; characteristic threads were seen here only very infrequently.

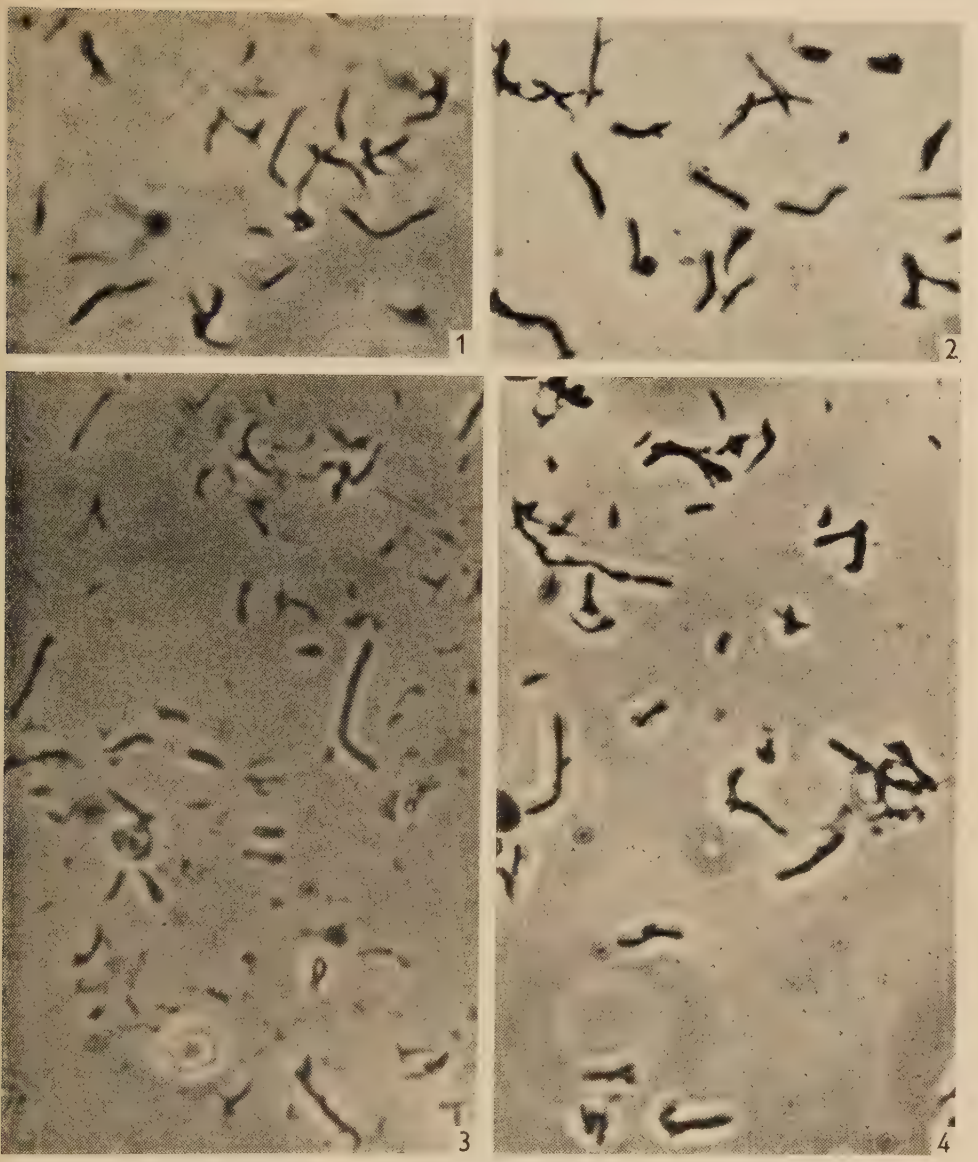
Fine structure in the pretreated materials generally emphasized and supplemented some of the evidence adduced above. For material treated for $\frac{1}{4}'$ and for 2′ with boiling water, no great grains in detailed structure were seen. A number of threads survived this abuse while retaining their general appearance. Apparently spewing and eroded threads and light particulate matter were often seen (Figs. 42 and 43). With the buffer-treated material, interesting detail was often apparent even though much of the material had the usual appearance after the treatments (*e.g.*, Fig. 44). The threads proved Feulgen and acetocarmine positive after 5′ and 20′ treatments at pH 8. Some displayed quite regular lateral structure (Figs. 45, 46, and 48 (shadow of lower thread)). Marked constrictions were again seen (Figs. 47 and 48 (upper thread)). Pairings or doubleness were evident in a number of instances (Figs. 49, 50, 51, and 52). Frequent indications of axial periodic structure (*e.g.*, Fig. 46) were most striking in some micrographs that showed virtual banding (Fig. 53) or a helical structure (Fig. 54).

LEGENDS FOR FIGURES

All micrographs are of chromatin threads prepared from chicken erythrocytes. Figures 1 to 4 are phase micrographs, the balance, electron micrographs.

Abbreviations: WB — Waring Blendor, LM — Logeman Mill, AA — acetic-alcohol, AC — acetocarmine, Cr — 2 per cent aqueous chromic acid, Os — 2 per cent aqueous osmium tetroxide, Sal Os — 2 per cent osmium tetroxide in saline, /B — bulk fixation, /v — vapor fixation, Untr — untreated, H_2O — treated with boiling water, Phos — treated with Sørensen buffer at pH 8.

	Means of Disruption	Fixation	Subject
Fig. 1	WB	AC/B	Range of forms
2	WB	AC/B	Range of forms
3	LM	AC/B	Range of forms
4	LM	AC/B	Range of forms
5	WB	AA/B	Relatively straight thd.
6	WB	Cr/B	ditto
7	LM	AC/B	ditto
8	WB	AA/B	Wavy thread
9	LM	Sal Os/B	ditto
10	WB	AA/B	V-shaped thread
11	LM	Sal Os/B	ditto
12	WB	Cr/B	Crescent-shaped thr.
13	LM	AC/B	ditto
14	WB	AA/B	J-shaped thread
15	LM	Sal Os/B	ditto
16	LM	Sal Os/B	ditto
17	WB	AA/B	Smooth ends
18	LM	AA/B	ditto
19	WB	AA/B	Tapered ends
20	LM	AC/B	ditto
21	WB	AA/B	"Tails"
22	WB	AC/B	ditto
23	LM	Os/v	ditto
24	WB	Untr	Bifurcated end
25	LM	Os/B	ditto
26	WB	Untr	Double body
27	LM	AC/B	ditto
28	WB	Os/B	Lateral structure
29	LM	AC/B	ditto
30	WB	Untr	Constrictions
Fig. 31	WB	AA/B	ditto
32	WB	AA/B	ditto
33	LM	AC/B	ditto
34	LM	Sal Os/B	ditto
35	WB	Cr/B	Recurrent crescent
36	WB	Cr/B	ditto
37	WB	Untr	Recurrent crook
38	WB	Untr	ditto
39	LM	AC/B	Disrupting,processed nucleus
40	LM	AC/B	ditto
41	—	Untr	Disrupting nucleus from untreated preparation of washed cells
42	WB	H_2O ¼′ AC/B	"Spewing" thds
43	WB	H_2O ¼′ AC/B	ditto
44	WB	Phos 20′ AA/B	Usual threads
45	WB	Phos 20′ AC/B	Lateral struct.
46	LM	Phos 20′ AC/B	ditto
47	WB	Phos 5′ AC/B	Constriction
48	LM	Phos 20′ AC/B	ditto
49	WB	Phos 20′ AA/B	Pairing of thds.
50	WB	Phos 20′ AA/B	ditto
51	LM	Phos 5′ AA/B	ditto
52	LM	Phos 5′ AA/B	ditto
53	LM	Phos 20′ AC/B	Banded appearance
54	LM	Phos 20′ AC/B	Helical appearance


1
2
3
4
10 μ

5
6
7
8
9
12
10
11
13
14
17
19
21
15
18
22
16
20
24
25
26
27
23
28
32
30
31
33
29
34
35
37
38
1μ
41
36
39
40

To permit quantitative comparisons, many threads were measured. It was found that, judging from the reasonably narrow ranges of width, length, and axial ratio, the use of various fixatives or pretreatments had no gross dimensional effects. For brevity, the assembled data will be given without reference to fixation or to pretreatment. For the Waring Blendor material, the following results obtain:

Run H: LMGs (Light-micrographs): 45 threads: average 0.4 × 6 micra, axial ratio $r = 15$, ranges 0.2–0.8 × 2–13, $r = 6$–38

Run H: EMGs (Electron-micrographs): 165 threads: ranges 0.09 0.83 × 1.4 - 15, $r = 4$–44

Run K: EMGs: 329 threads: ranges 0.07–0.80 × 0.8–8.9, r = 3–49.

Arrays of the lengths of these 494 threads from the electron-micrographs (Table I) proved to be unimodal at *ca.* 3 micra (Textfig. 1).

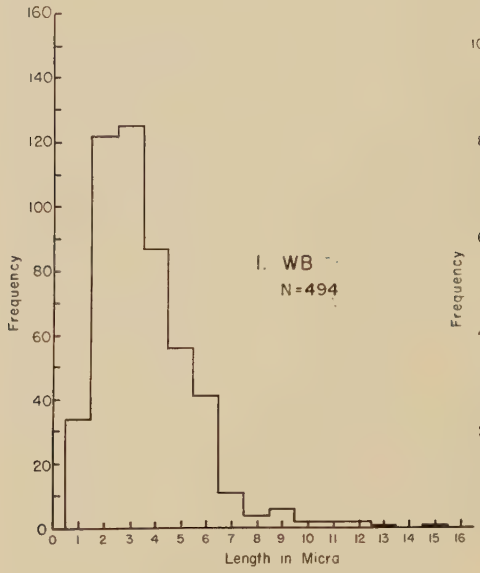


Fig. 1.

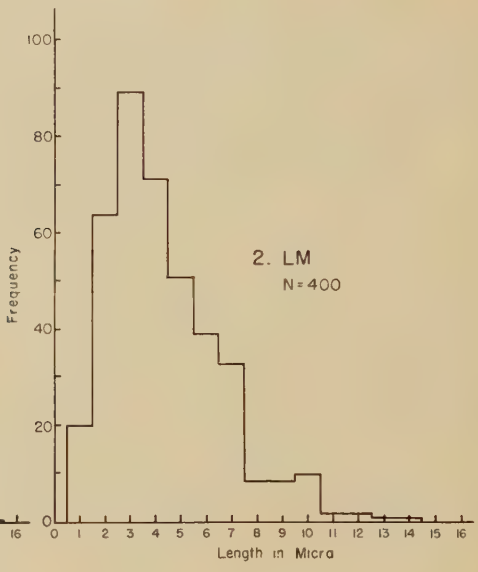


Fig. 2.

Fig. 3.

Fig. 4.

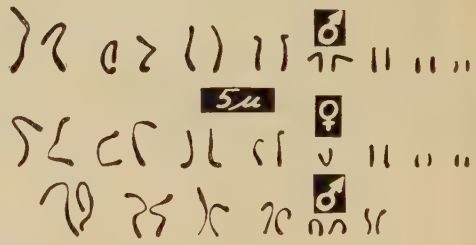


Fig. 5.

Textfigs. 1 and 2. Frequency distributions of lengths of chromatin threads: **1** with the Waring Blendor, **2** with the Logeman Mill.
Textfigs. 3 and 4. Metaphase plates of chicken chromosomes, from Matthey (7) after Yamashina (18).
Textfig. 5. Arrays of macrochromosomes of *Gallus domesticus* from Matthey (7) after Oguma (13) and Yamashina (18).

For the material prepared by the Logeman Mill, the following obtain:

Run I: LMGs: 30 threads: average 0.5 × 6 micra, $r = 15$, ranges 0.2–1 × 2.1–11, $r = 4$–55

Run I: EMGs: 111 threads, ranges 0.07–0.8 × 2–12, $r = 5$–55

TABLE I

Arrays of Lengths of Chromatin Threads Prepared with the Waring Blendor.

Length, micra	Run H	Run K	Sum
1	6	28	34
2	25	97	122
3	36	89	125
4	28	59	87
5	29	27	56
6	23	18	41
7	5	6	11
8	3	1	4
9	3	3	6
10	1	1	2
11	2	0	2
12	2	0	2
13	1	0	1
14	0	0	0
15	1	0	1
16	0	0	0
	165	329	494

TABLE II

Arrays of Lengths of Chromatin Threads Prepared with the Logeman Mill.

Length, micra	Run I	Run J	Run K	Sum
1	0	4	16	20
2	9	14	41	64
3	17	31	41	89
4	28	29	15	72
5	18	25	8	51
6	17	19	3	39
7	12	17	4	33
8	5	3	0	8
9	2	5	1	8
10	2	6	2	10
11	0	2	0	2
12	1	1	0	2
13	0	1	0	1
14	0	1	0	1
	111	158	131	400

Run J: LMGs: 116 threads, average 0.3 × 5.5 micra, $r =$ 18, ranges 0.2–0.8 × 2–9, $r = 4$–45
Run J: EMGs: 158 threads, ranges 0.08–0.8 × 1–14, $r = 4$–59
Run K: EMGs: 131 threads, ranges 0.05–0.6 × 1–10, $r = 4$–46.

Arrays of lengths of the 400 threads from the electron-micrographs (Table II) also proved unimodal at 3 micra (textfig. 2). Totalling the data from the electron-micrographs, one sees the following:

Waring Blendor: 494 threads, range roughly 0.1–0.8 × 1–15 micra, $r = 4$–50;
Logeman Mill: 400 threads, range roughly 0.1–0.8 × 1–14 micra, $r = 4$–60.

A common order of size is thus seen for all the runs and for the two methods of preparation. This correspondence is emphasized by the histograms of Textfigs 1 and 2. These are both unimodal with slight positive skewness, and show similar extent, kurtosis, and modes at 3 micra.

TABLE III

Measurements of Metaphase Macrochromosomes of *Gallus Domesticus* from Published Plates.

Macrochromosome Number	Length in Micra: From reference no. (17)	(16)	(8)	(13)	(18)	Mean	Width in Micra: From reference no. (17)	(16)	(8)	(13)	(18)	Mean
1	6.2	6.9	4.7	6.6	7.1	6.3	0.4	0.5	0.5	0.25	0.4	0.41
2	4.5	4.9	3.2	5.0	5.4	4.6	0.37	0.4	0.5	0.33	0.3	0.38
3	2.4	3.9	2.6	3.8	3.8	3.3	0.33	0.5	0.5	0.25	0.4	0.40
4	1.9	3.5	1.9	3.5	3.3	2.8	0.34	0.45	0.4	0.25	0.3	0.35
5	1.4	3.2	2.0	2.6	3.4	2.5	0.34	0.5	0.5	0.25	0.4	0.40
6	1.1	2.3	1.5	2.2	2.2	1.9	0.34	0.4	0.4	0.25	0.3	0.34
7	0.8	—	0.9	—	1.8	1.2	0.29	—	0.3	—	0.3	0.30
8	0.6	—	—	—	1.5	1.0	0.24	—	—	—	0.3	0.27

Macrochromosome Number	Approximate Axial Ratios: From reference no. (17)	(16)	(8)	(13)	(18)	Mean
1	16	14	9	26	18	16
2	12	12	6	15	18	12
3	7	8	5	15	9	9
4	6	8	5	14	11	9
5	4	6	4	10	8	6
6	3	6	4	9	7	5
7	3	—	3	—	6	4
8	2.5	—	—	—	5	3.8

Average deviations from mean widths and lengths are *ca.* 20 per cent.
References in order: White (17).
Unger (16).
Miller (8).
Oguma (13).
Yamashina (18).

DISCUSSION

The chromosome complement of the domestic chicken (Textfigs. 3 and 4) is cytologically characterized by great range in size and form as well as by high number (7). The 8 pairs of macrochromosomes include large V's with

unequal arms, large rods, smaller V's with unequal arms, small V's with equal arms, and short rodlets (Textfig. 5); the 31 pairs of microchromosomes appear in the light microscope as a "dust" of objects extending to the limit of visibility. Qualitatively we see, then, the range of forms encountered in our isolated materials.

For quantitative purposes we have measured metaphase plates published by several authors (Table III). The range of length of the macrochromosomes here agrees well with the results given in the foregoing. It is noteworthy that most of these are 2 to 3 micra long, corresponding to the mode seen in the distributions given above. The widths and the axial ratios here also lie in the ranges seen in the foregoing; higher ratios seen from the electron-microscopy are to be expected from the smaller widths measurable with that instrument. The quantitative similarity of the isolated and mitotic materials is thus very striking. This unexpected correspondence may represent a special case for this nonmitotic cell. However, it may simply indicate that the isolated material responds to the artificial ionic environment by assuming a "mitotic" form.

The similar results obtained after use of the Waring Blendor and of the Logeman Mill are felt to be good evidence that these chromatin threads were indeed preformed structures rather than random derivatives of nuclei. It might be argued that the constancy of the necessarily controlled physical conditions in these preparations would fractionate similar material. However, the preliminary examination of the supernatant suggested that bodies corresponding to the macrochromosomes are apparently well separated from smaller bodies by the centrifugation used, so that we should be addressing good representations of these threads. Further, the detailed similarity found in the threads by two grossly different physical means and their mutual qualitative and quantitative resemblance to mitotic chicken chromosomes seem of considerable cogency.

SUMMARY

In the first of a series of experiments on the identity of isolated chromosomes, chromatin threads from chicken erythrocytes disrupted by two radically different mechanical means have been compared. The Waring Blendor and the Logeman Hand Colloid Mill have been used. The Blendor combines great turbulence and shear; the Mill applies relatively mild, pure shear. Remarkably similar results were obtained by these two divergent techniques. Phase-microscopic study of wet mounts after various treatments

indicated progressive and successive disruption of cells and of nuclei to form characteristic threads that stained well with three nuclear dyes including the Feulgen reagent. These chromatin threads displayed common ranges of form and size that are remarkably congruent with the cytological picture of metaphase macrochromosomes of the chicken. In addition, they possessed structural features signally associated with chromosomes, including doubleness, coiled aspect, individuality as shown by recurrence, and longitudinal differentiation as shown by constrictions and by axial periodic structure. These results support the identification of such isolated chromatin threads with the chromosomes.

The author acknowledges with gratitude his substantial indebtedness to a number of colleagues. He is especially indebted to Dr. John J. Biesele for encouragement and innumerable helps; to Mr. Louis Hildebrand for extensive general assistance; to Miss Grace A. Cohn for photographic services; and to Mr. Mark E. Gettner for help with the electron microscope.

REFERENCES

1. Calvet, F., Siegel, B. M., and Stern, K. G., *Nature*, **162**, 305 (1948).
2. Claude, A., *Trans. N. Y. Acad. Sci.*, **4**, 79 (1942).
3. Claude, A., and Potter, J. S., *J. Exptl. Med.*, **77**, 345 (1943).
4. Denues, A. R. T., *Exptl. Cell Res.*, **3**, 388 (1952).
5. Lamb, W. G. P., *Nature*, **164**, 109 (1949).
6. —— *Exptl. Cell Res.*, **1**, 571 (1950).
7. Matthey, R., Les Chromosomes des Vertébrés. F. Rouge, Lausanne, 1949.
8. Miller, R. A., *Anat. Rec.*, **70**, 155 (1938).
9. Mirsky, A. E., and Pollister, A. W., *Trans. N. Y. Acad. Sci.*, **5**, 190 (1943).
10. —— *Biological Symposia*, **X**, 247 (1943).
11. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, **31**, 1 (1947).
12. —— *ibid*, **34**, 475 (1951).
13. Oguma, K., *Annot. Zool. Japonenses*, **17**, 612 (1938).
14. Ris, H., and Mirsky, A. E., *Exptl. Cell Res.*, **2**, 263 (1951).
15. Ruch, F., *Chromosoma*, **3**, 357 (1949).
16. Unger, H., *Z. Zellforsch. u. mikroskop. Anat.*, **25**, 476 (1936–7).
17. White, M. J. D., *J. Genetics*, **26**, 345 (1932).
18. Yamashina, M. Y., *Cytologia*, **13**, 270 (1944).

STUDIES ON THE STRUCTURE OF MUSCLE

II. ARTHROPOD MUSCLES[1]

J. L. FARRANT and E. H. MERCER

Chemical Physics Section, Division of Industrial Chemistry, C. S. I. R. O., Australia

Received September 25, 1951

The electron microscope has been used to study the fine structure of striated muscles from several species of animals, but so far nothing seems to have been reported concerning arthropod muscles, although these, on account of the length of their sarcomeres and clarity of banding, have provided histologists with exceptionally favourable material from which much has been learned concerning the phenomenon of contraction. The work to be described in this paper was initiated with the object of ascertaining whether these muscles possess similarly valuable properties from the point of view of electron microscopy. Two representative types of muscle, the wing muscles of a grasshopper (*Locusta migratoria* L.) and the leg muscle of a crab (*Paragrapsus Quadridentatus*), have been studied by a technique involving the mechanical disintegration of whole muscle into fragments fine enough for electron microscopy. The two types of muscle differ widely in their speeds of action: crab muscles are slow compared to the wing muscles which are notable for their rapidity of action.

Preliminary examination showed that both kinds of muscle were readily broken down mechanically into fine fibrils (myofibrils) in which Z and A bands could be distinguished, but that the density of these fibrils to 50 KV electrons was so high that micrographs of intact fibrils as clear as those obtained from toad muscle (4) or rat muscle (5) could not be obtained. However it was found possible to disrupt the myofibrils further and thus to free the component myofilaments, the probable contractile units of the muscle, in forms suitable for study. Particular attention was therefore paid to these structural elements.

Following the lead given by Szent-Györgyi (12) the contractile properties of muscle are at present attributed to the actomyosin complex and it is important, in order to establish a relation between morphology and biochemistry, to locate histologically each of the proteins, myosin and actin. Actin,

[1] The paper by Draper and Hodge (4) is to be regarded as Part I of this series.

isolated by Straub's methods (10, 11), possesses to a remarkable degree the power of forming fibers by what appears to be a linear association of particles (6, 7). These fibrils of actin resemble in some respects the myofilaments of muscle, and this fact has led Rozsa, Szent-Györgyi and Wyckoff (7) to suggest that an actin fibril may form the basic element of the actomyosin filament and that the myosin is attached to the surface of the actin fibril. The isolated filaments of crab and insect muscle were found useful for examining this question and the results lend support to the above hypothesis. Particular attention was paid to evidence of a duplex structure in the myofilaments and of periodicities in the basic fibril which would lead to an identification of these with actin. Under some circumstances artificial actin fibrils appear to exhibit a periodic nodulation of the order of 300 A (7).

PREPARATION OF MATERIALS AND ELECTRON MICROSCOPY

The electron microscope used in this work was an R.C.A. Type EMU which has been modified in this laboratory. Standard methods of mounting specimens and of metal shadowing were used (13).

Suspensions of disintegrated muscle suitable for microscopy were prepared by the methods described by Draper and Hodge (4). Muscles were removed immediately after the death of the animal and were used either without fixing, or fixed in formol saline (5 per cent formaldehyde in normal saline). After fixing for 24 hours (or immediately when not fixed) the muscles were comminuted in a Waring Blendor until a homogeneous aqueous suspension resulted from which the coarser fragments were removed by centrifugation. This suspension formed the basis of special preparations.

From the limited survey yet made of the musculature of various animals it is clear that muscles from different sources differ in the ease with which they can be disrupted into myofibrils by this procedure. Toad muscle is readily reduced to fragments small enough for electron microscopy; rabbit and rat muscle are somewhat tougher. The arthropod muscles examined were very difficult to break down beyond relatively coarse fibers (ca. 100 μ wide) and, when finer fibrils resulted, these appeared in the electron microscope as dense rod-like forms. A short digestion with trypsin (a Difco Preparation), pH 7, 40° C, 10–30 minutes) was found useful to soften these coarse fibrils. Mechanical disintegration with water of the digested material then released the contents of the dense fibrils in various degrees of disintegration. Fibrils resulting from mechanical and enzymic attack are shown in Fig. 1 (crab muscle) and 2 (grasshopper wing muscle). In the crab muscle the remnants of Z and A bands can be seen; in the wing muscle the Z bands alone remain. The continuity of the filaments through the Z bands is well

demonstrated. The greater sarcomere length is appreciated by comparing these E.M.G.'s with those of toad muscle. (Toad muscle 2.5 μ, crab muscle 5 μ, insect muscle 4 μ). The resistance of arthropod muscles to disintegration is not due to the presence of a strong sarcolemma sheath reinforced by fibrous connective tissue as in vertebrate muscle. Thin sheets, but no connective (collagen) fibrils, were observed in these preparations. The coherence of these fibers and their density appears to be due to large amounts of "accessory proteins" associated with the fibrils. Snellman and Gelotte (9) pointed out that even rabbit muscle is very dense when the soluble proteins, etc., are not washed out. The relative transparency of the toad muscle fibrils, described by Draper and Hodge, may have been due to the removal of much soluble material during their preparation.

It was found that the "accessory proteins" could be removed from fresh unfixed muscle by salt solutions. Experiments were accordingly made to determine the location of the salt-soluble protein in insect muscle. Unfixed wing muscle was extracted for a few minutes with 0.9 per cent sodium chloride solution, dispersed in water and fixed by addition of formalin. Partly disintegrated myofibrils were observed such as those illustrated in Fig. 3. In this preparation there is at A a portion of intact fibril, at B some extracted material, which has formed a fibrous precipitate, and at C the filamentous residue. Since the "accessory protein" is soluble in saline solutions it may be regarded as a "myosin." From formalin-fixed wing muscles the same protein is removed by trypsin leaving a preparation of very clean filaments held together by the remnants of Z-bands. (See Figs. 2 and 6.) Such micrographs seem to show that the wing muscle fibril consists of a core of fine filaments embedded in the "accessory protein" to give the dense rodlet.

Snellman and Gelotte (9) have extracted rabbit muscle with 0.3 M KCl and noted electronmicroscopically the removal of the isotropic bands. Since the A-band substance may have protected the fibrils associated with it from dissolution, it does not follow from this observation that the myosin is mainly localized in the isotropic region nor that there is necessarily disagreement with the present work. The A band substance has previously been noted to protect the underlying filaments of toad and crab muscle from enzymic attack and to hold them together in sheaf-like bundles.

Similarly the dense rodlet myofibrils of disintegrated crab muscle yielded to tryptic digestion and broke down into extensive sheets of filaments (Fig. 1) with a well developed lateral association. The relation of these sheets to the original dense fibrils was not clear. Further digestion combined with fractional

sedimentation led to preparations consisting almost entirely of freed filaments. The fine structure of these filaments, when stained with phosphomolybdic acid and when shadowed, will be discussed in the next section.

DISCUSSION

Electron micrographs of crab and insect muscle prepared by the present methods do not add greatly to our knowledge concerning the larger structural features of the myofibrils except to emphasize, in agreement with Snellman and Gelotte (9), that considerably larger amounts of electron scattering material may be associated with the fibrils than is suggested by the E.M.G.'s of fixed and washed muscle. As mentioned above the exceptional clarity of the micrographs of toad muscle may be due to the easier removal of this material by washing. The distribution of A-band and Z-band material in crab and insect muscle may have been disturbed by tryptic digestion and mechanical disruption and this needs to be borne in mind when considering an E.M.G. such as Fig. 1, which does not show a precise localization of A and Z bands.

The principle interest at the present time concerns the characterization of the various features revealed by electron microscopy and their identification with proteins extracted by biochemical procedures. The common elements discovered in all the striated muscle examined electron microscopically (toad, rat, rabbit and the present examples) are: (a) the fine continuous filaments of indefinite length but constant width. The width of these filaments is not the same in all types of muscles, e.g. toad muscle 120 A, insect wing muscle 70 A and crab leg muscle 250 A[1], and it is an interesting question whether filament width is related to speed of response or other properties of these muscles. (b) Z-band protein or protein complex, (c) A-band protein etc., (d) the less precisely localized "accessory protein" distributed between and enveloping the filaments.

The proteins (or protein) referred to here as "accessory" may be divided into two parts on the basis of their ease of removal from the myofibril. The greater part appears easily digested by enzymes in fixed arthropod muscle or extracted by salt solutions from material which has not been fixed. A smaller portion closely invests the myofilaments, serving to link them together laterally. This fraction, by reason of its regular distribution, is probably responsible for the appearance of a periodic nodulation (400–450 A) in toad and rat muscle and may also contain those non-volatile

[1] Diameter of the compound filament to be discussed later in the text.

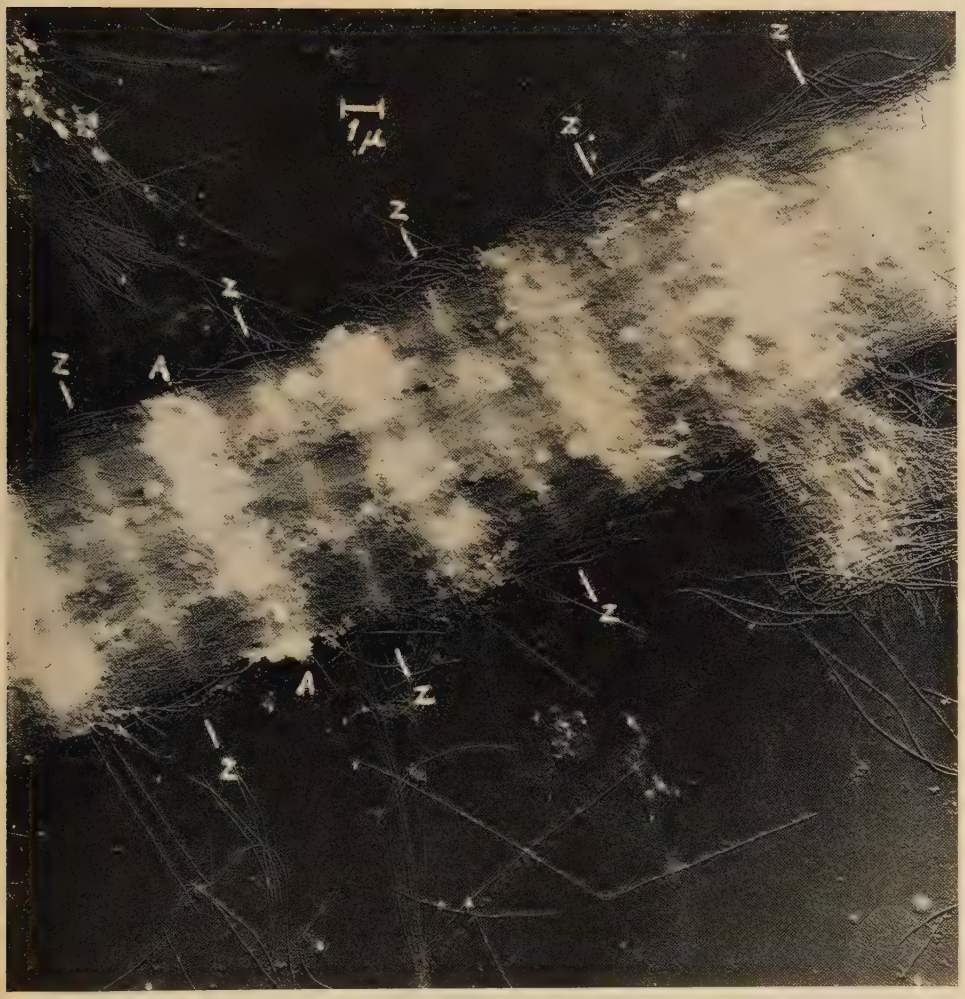


Fig. 1. Electron micrograph of fragment of crab leg muscle, formalin fixed, and digested briefly with trypsin. Shadowed with uranium. Shows extensive sheets of parallel myofilaments overlaid with remnants of A and Z bands.

inorganic constituents which remain when the fibril is incinerated in the electron beam (3). Neither insect muscle nor crab muscle was observed to display as definite a periodic distribution as toad muscle, although a quasi-regular distribution can be seen in such a micrograph as Fig. 4. A less obvious and finer nodulation was sometimes noted in the filaments of insect muscle after enzymic digestion.

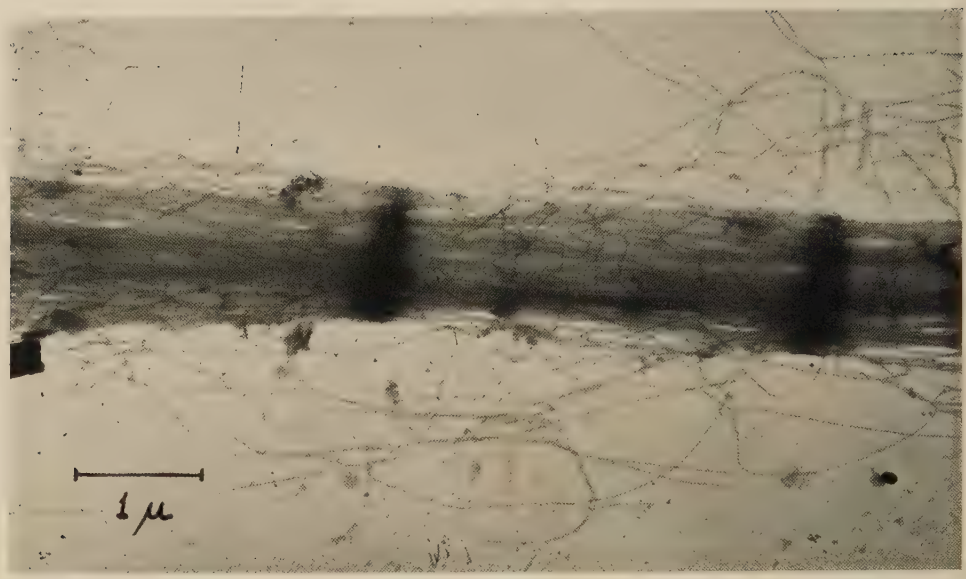


Fig. 2. Fragments of grasshopper wing muscles, formalin fixed and digested with trypsin until only Z-bands remain. Stained with phosphomolybdic acid. Note continuity of myofilaments through Z-bands.

The observation, that the "accessory protein" of insect muscle is soluble in solutions of salt, suggests that this material represents the myosin-type protein in these muscles. Were this the case it would seem that (at least in this instance) the basic fibrillar frame work (visible at C in Fig. 3) represents the actin and that the myosin fraction is associated more or less loosely with the bundle of actin fibrils. This disposition of myosin and actin agrees with that proposed by Rozsa, Szent-Györgyi and Wyckoff (8) and by Snellman and Gelotte (9).

A further characteristic, which may support this view, is the peculiar duplex structure of the filaments derived from formalin-fixed crab muscles. This may be seen both in shadowed specimens, where patches of material may be seen adhering to the fibril, which itself appears as a distinct and separate structural element (see Fig. 4), and also in phosphomolybdic stained specimens, where the same adhering material appears to stain more deeply. In addition to the diffusely adhering material, which takes the stain, the outer layers of the fibril itself stain more deeply causing them to appear tubular as can be seen in Fig. 5. The tubular appearance would be expected if the phosphomolybdic acid stains only the peripheral layers of the fibril or if

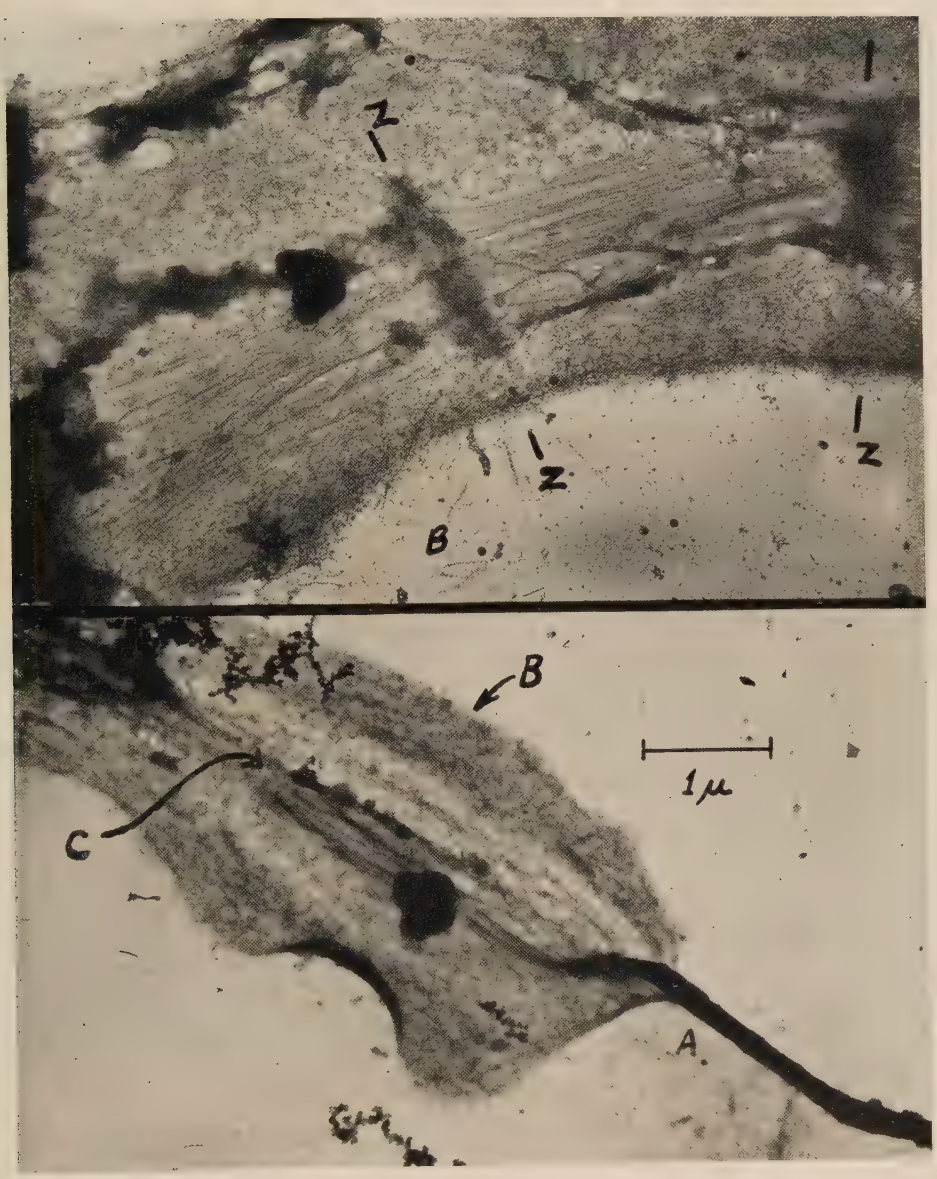


Fig. 3. Results of extracting unfixed insect wing muscle with 0.9 per cent sodium chloride. A—intact dense myofibril; B—extracted protein (myosin?); C—filamentous core as shown also in Fig. 2 (actin?).

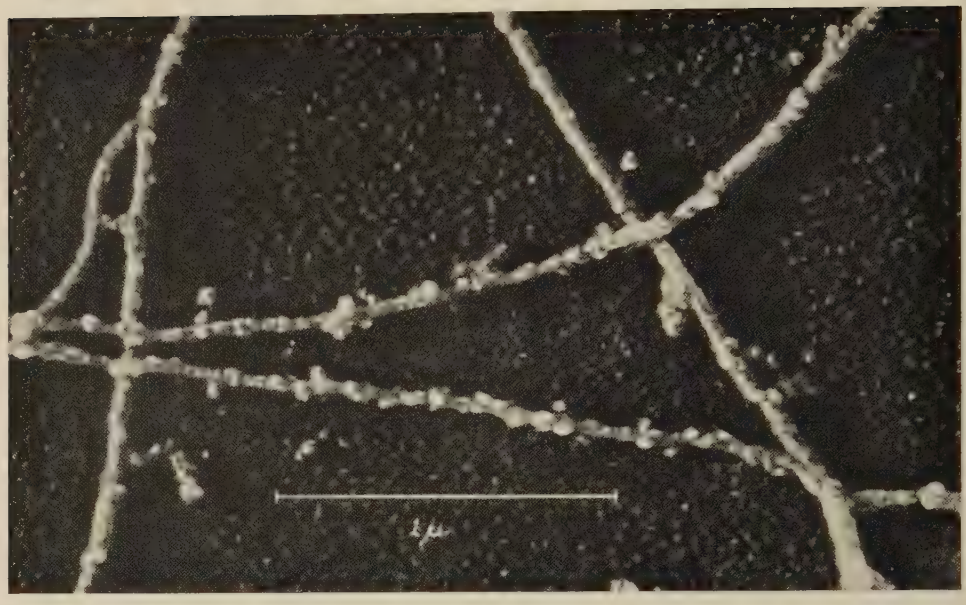


Fig. 4. Myofilaments of crab muscle showing the basic fibril and adhering protein. Shadowed with uranium.

it does not penetrate the interior of the fiber. We reject the latter hypothesis, since the tubular appearance is equally well defined in all stained fibrils whether they have been exposed to the stain (0.2 per cent phosphomolybdic acid solution pH 4.5) for only 5 minutes or some weeks, and think that the filament actually consists of a core (diameter 160 A) of a lightly staining protein and a sheath (40 A) of a protein having a greater affinity or alternatively the fibrils are hollow tubes. There was no evidence that either protein formed a spiral as has been suggested by Bennett (2).

Accepting the assumption that the "accessory proteins" represent the myosin component of these muscles, it is clear that much of it is loosely bound and not very obviously oriented as a fibre. The absence of orientation may of course arise solely as a consequence of the method of preparation and not be an original feature, but on the face of it there appears to be some disagreement here with the results of X-ray examination of muscles, which reveals a fiber type orientation of the myosin (shown by the characteristic 5.1 A meridonial arc of the α-proteins) as well as of the actin component (1).

In fibrils of actin formed artificially Rozsa *et al.* (7) noted a periodicity of

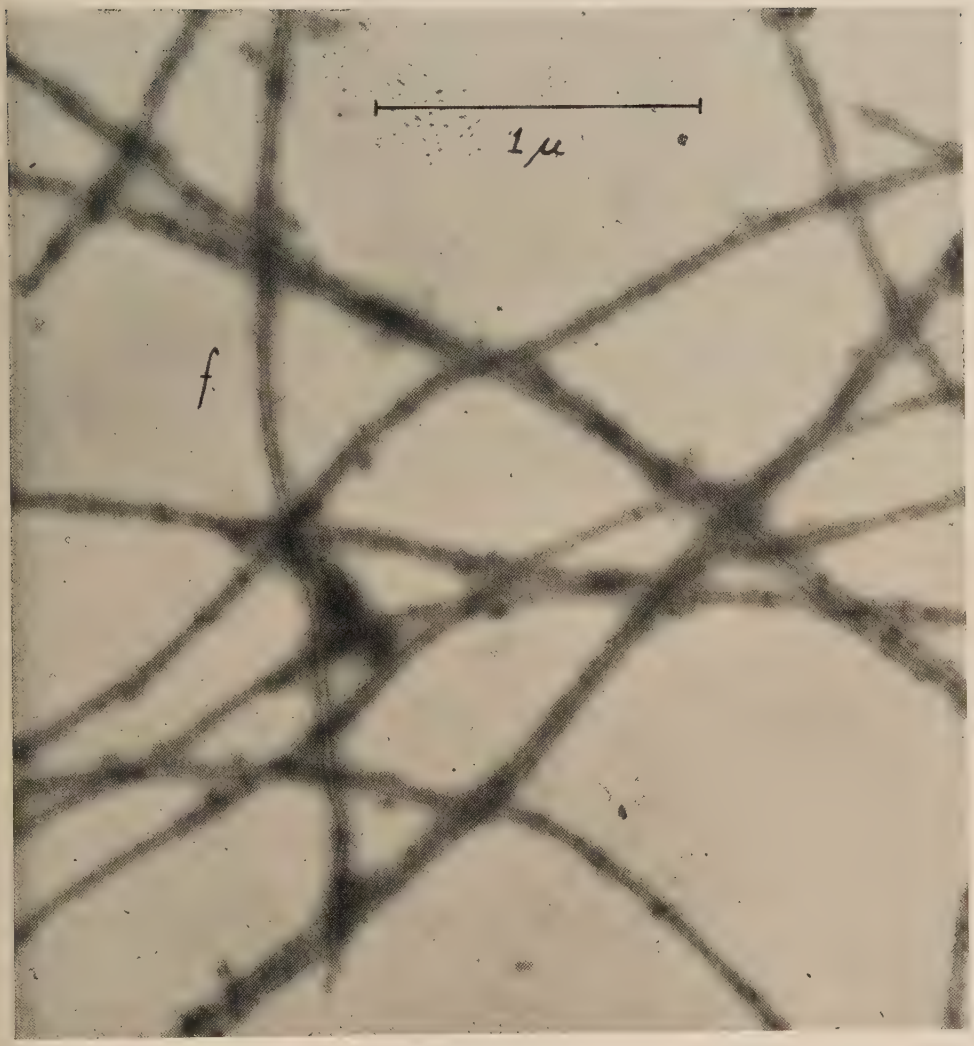


Fig. 5. As for Fig. 4. Filament stained with phosphomolybdic acid. Compare with Fig. 4. The filament marked f appears to consist of a central core of diameter ca 160 A and an enveloping sheath 40 A thick. See Text.

the order of 200–300 A. No feature of this order of magnitude was visible in the fibrils which form the basic components of the filaments derived from insect or crab muscles. The periodicity in filaments of rabbit muscle, to which these authors draw attention, seems rather to be due to the regular

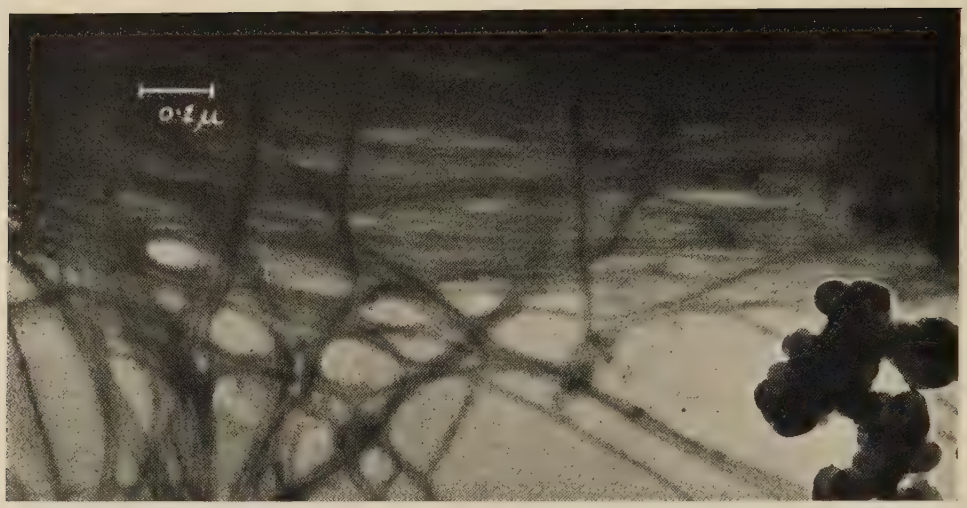


Fig. 6. Filaments from grasshopper wing muscle cleaned by tryptic digestion and stained with phosphomolybdic acid. High resolution micrograph showing absence of nodulation on filaments. Diameter ca 70 A.

manner in which portion of the "accessory protein" (myosin?) is distributed along the length of the basic fibril. High resolution micrographs (resolution ca. 20 A) of the cleanest filaments obtained in this investigation (insect muscle fixed with formalin and digested with trypsin) showed that they were extremely smooth, uniform and featureless (Fig. 6). (Diameter ca. 70 A.) It seems certain that any external unevenness as large as 200–300 A would have been revealed in these micrographs. If it is assumed that these fibrils do consist of actin, then it seems that a smaller actin particle must participate than that appearing in the micrographs of Rozsa *et al.* of artificially formed fibrils.

SUMMARY

An electron microscopical examination of mechanically disintegrated insect wing muscle and crab leg muscle shows that these arthropod muscles are broken down into relatively dense myofibrils in which A and Z bands may be distinguished.

After tryptic digestion crab muscle fibrils can be further resolved into sheets of filaments with a strong lateral association; insect muscle similarly treated resolves into bundles of filaments held together by the remnants of

the Z-bands. Further digestion releases free filaments in a form suitable for high resolution study.

The density and coherence of the myofibrils seems to be due to the presence of an "accessory protein" which may be extracted by salt solutions from insect material leaving behind a bundle of filaments. It is possible that the proteins soluble in salt solution represent the myosin fraction of the muscle and therefore that the fibrillar remnant is the actin. This picture of the muscle fiber as consisting of a basic fibrillar frame work to which the myosin is attached is in agreement with the suggestions made by Rozsa, Szent-Györgyi and Wyckoff.

Stained and shadowed specimens of single isolated filaments of crab muscle seem to consist of a core of one protein which is enveloped by a second protein. It is suggested that the quasi-periodic nodulation of the filaments of these arthropod muscles and also the more regular nodulation of toad, rat, etc. muscles is due to the distribution of the accessory protein (myosin?) along the basic fibrils (actin?). The basic fibrils themselves, unlike those of certain kinds of artificially formed actin fibrils are smooth and structureless.

REFERENCES

1. Astbury, W. T., *Proc. Roy. Soc.* (*London*), B **134**, 303 (1947).
2. Bennett, H. S., *Anat. Rec.*, **103**, 423 (1949).
3. Draper, M. H., and Hodge, A. J., *Nature*, **163**, 576 (1949).
4. —— *Austr. J. Exptl. Biol. Med.*, **27**, 465 (1949).
5. Hall, C. E., Jakus, M. A., and Schmitt, F. O., *Biol. Bull.*, **90**, 32 (1946).
6. Jakus, M. A., and Hall, C. E., *J. Biol. Chem.*, **167**, 705 (1947).
7. Rozsa, G., Szent-Györgyi, A., and Wyckoff, R. W. G., *Biochim. et Biophys. Acta*, **3**, 561 (1949).
8. —— *Exptl. Cell Res.*, **1**, 194 (1950).
9. Snellman, O., and Gelotte, B., *ibid*, **1**, 234 (1950).
10. Straub, F. B., *Studies Inst. Med. Chem. Univ. Szeged*, **2**, 3 (1942).
11. —— *ibid.*, **3**, 23 (1943).
12. Szent-Györgyi, A., Chemistry of Muscular Contraction. New York, Academic Press, 1947.
13. Wyckoff, R. W. G., Electron Microscopy. Interscience, New York, 1949.

THE EFFECT OF ELECTROLYTES ON THE PHYSICAL STATE OF THE NERVE AXON OF THE SQUID AND OF STENTOR, A PROTOZOON

ROBERT CHAMBERS and CHIEN-YUAN KAO

Lilly Research Laboratories, Marine Biological Laboratory, Woods Hole, Mass.

Received September 26, 1951

Recently, Hodgkin and Katz (6) in their studies on the giant nerve of the squid found that solutions of various alkaline earth salts act in a manner to indicate that the salts of the divalent cations disperse the material composing the interior of the axon, while those of monovalent cations do the reverse. These results led them to refer to similar findings reported in the literature concerning the protoplasmic interior of several types of cells exposed to these salts.

It is generally accepted that solutions of the alkaline earth salts affect the physical state of the protoplasm of cells, also that salts of monovalent and of divalent cations act oppositely, one inducing solidification and the other, liquefaction. The controversial feature has been which of the two types of ions has the solidifying and which, the liquefying effect. Heilbrunn and associates (5) working with the centrifuge method of testing cytoplasmic viscosity have claimed that chlorides of the divalent cations exert a liquefying action on the protoplasmic interior. Such an effect is surprising on the basis that the continuous aqueous phase of the protoplasmic interior of the many cells tested has a pH close to neutrality at which the contained ionizable proteins are generally on the alkaline side of their iso-electric points. The proteins should therefore act as proteinates of which their calcium salts would have a lesser tendency to disperse than the corresponding sodium or potassium salts.

There have been chiefly two methods for determining the action of chemical agents on the physical state of the protoplasmic interior of living cells. One is to expose the cells to a given solution and to note the effect on displacement induced by centrifugation of visible cytoplasmic structures or granules. The other method, the micromanipulative, is more direct and consists of noting the resistance to microneedles of the interior of cells immersed in given

solutions or of noting the effect of microinjecting the solutions directly into the cell (1). Both methods have been used and from them have developed two divergent points of view regarding the action of salts of divalent and of monovalent cations on the protoplasmic interior. With the micromanipulation method, the effect reported has been uniformly that the chlorides of the divalent cations induce either irreversible or reversible coagulation while those of monovalent cations, if anything, decrease the consistency of the protoplasm. The cells which have exhibited these responses have been frog muscle fibers in Ringer's solution, echinoderm eggs in sea water, certain fresh water protozoa, e.g., the amoeba and the protoplast of root-hair cells of the fresh water plant, *Limnobium* (1, 7).

The centrifugation method of testing the effect on the cell interior by exposing cells to various salt solutions has the disadvantage of uncertainty. There is no knowing how much of a given salt penetrates, if at all. Moreover, in cases where penetration does occur the salt may have an action on the cellular inclusions themselves, the displacement of which the investigator depends upon for determining the viscosity of the cell interior.

Hodgkin and Katz performed their experiments on the axon of the giant nerve surgically isolated from the mantle of the squid. They squeezed the contents of the axon through the cut ends of fragments of the fiber into a solution of 0.58 M NaCl (isotonic with Plymouth sea water) and found that the extruded material retained its form and reacted as if it were a jellied mass. They then transferred cut-off pieces of these jellied columns to dishes containing different concentrations of various salt solutions. They found that in solutions of NaCl and KCl, the material remained in a jellied state but became dispersed in solutions of Ca, Sr, Ba or $MgCl_2$. They found that 0.39 M solutions of $CaCl_2$ (isotonic with Plymouth sea water) induced dispersion in seven minutes while the chlorides of the other divalent cations did so only after thirty-five minutes. They regarded the dispersive action of $CaCl_2$ to be specific, although they state that, with $MgCl_2$, the immersed axoplasm gradually lost its sharp boundaries without, however, losing its identity.

EXPERIMENTS

A. *Stentor.* In referring to the literature, Hodgkin and Katz made mention of immersion experiments performed on Stentor, a protozoon which gave results on the physical state of the Stentor in harmony with theirs on the axon. Heilbrunn (5) by centrifuging Stentor immersed in solutions of various salts, obtained results which made him conclude that salts of divalent cations, e.g.,

Ca, decrease, while those of the monovalent cations, Na and K, increase the viscosity of the protoplasmic interior.

In view of the reported experiments we tested his results by using the micromanipulative method on Stentor. Microinjections were made of solutions of $CaCl_2$ and of $SrCl_2$ in concentrations used by Heilbrunn in his immersion experiments, viz., 0.017 M and 0.034 M. The organisms were captured in spring water by impaling them on the end of a coarse microneedle, an operation which created no obvious permanent damage. The microinjections were performed with the tip of a micropipette thrust deep into the interior of the Stentor. The aperture of the micropipette averaged 2 to 3 micra in diameter and the amount injected varied in volumes of about 10 to 20 cubic micra to a volume which caused a considerable increase in size of the organism. The Stentor used was of a large variety. It readily changes its shape from that of a trumpet to a sphere. When spheroidal it averages about 200 micra across. In every case the reaction to the injection was an immediate and rapid clotting, localized at the site of the injection. The clot was quickly moved to the surface and was pinched off, in a manner similar to that described for the amoeba (4). After loss of the extruded clot, the Stentor behaved and appeared normal. Even when a considerable amount of the interior had been thus affected by the injected solution so that as much as two thirds of the interior had been coagulated and ejected (including one to several separated nuclei), nevertheless, the cortex of the organism persisted undisturbed. The surviving Stentor, except for its diminutive size, maintained its characteristic trumpet-shape and appeared normal.[1]

Dissection experiments also were done on Stentors. When caught and torn open in spring water, there was little if any extrusion of the interior. The tear rapidly closed and the Stentor continued to swim about in a more or less deformed shape depending upon the degree of the tear inflicted.

The Stentors were also immersed in 0.017 M and 0.034 M solutions of $CaCl_2$ or $SrCl_2$. In both of these solutions the Stentors swam around actively and appeared much as they do in spring water. However, the slightest tearing of the surface of a Stentor immersed in either of the solutions of calcium or of strontium chloride resulted in an immediate gaping of the tear and a clotting of the exposed endoplasm. If the tear was large, most of the interior became extruded and coagulated while the cortex underwent disorganization. If the tear was small, the coagulation of the extruding material

[1] In the course of the injection experiments phenol red and brom cresol purple as pH indicators were introduced into the Stentor. The phenol red readily diffused throughout, coloring the cytoplasm yellow and the string of nuclear nodes red. Brom cresol purple gave a uniform blue color. These indicate a colorimetric pH value of 6.8 for the cytoplasm and at least 7.6 for the nucleus.

impeded further extrusion, the ciliated cortex closed over the tear and the Stentor persisted living, though greatly distorted.

Dissections were also performed on Stentor immersed in 0.02 M and 0.05 M solutions of NaCl and of KCl. A tear of the surface was never repaired but resulted in an outflow and dissipation of the interior of the Stentor with no sign of coagulation.

These experiments place Stentor in the same category as *Amoeba dubia* in which $CaCl_2$, injected in concentrations of 0.017 M or higher, immediately induces localized coagulation followed by an extrusion and pinching off of the affected region, whereas NaCl and KCl do not.

B. *Nerve of the mantle of the squid.* Preliminary to our microinjection studies on the giant nerve fiber of the squid, we repeated Hodgkin and Katz' experiments of testing the effect of the various salt solutions on the extruded axoplasm. We confirmed their results and extended them to include higher concentrations of $CaCl_2$.

The giant nerve fiber from the mantle of the squid was surgically isolated in running sea water and transferred to a solution of 0.52 M NaCl isotonic with Woods Hole sea water. There it was cut into pieces, about 1 cm long, from which the axoplasm was extruded. The axoplasmic extrusions maintained their form in NaCl but underwent dispersion on being transferred to dishes containing $CaCl_2$. We used $CaCl_2$ also in concentrations of 0.34 M, 0.68 M, 1.36 M and 2.72 M and found that the dispersion occurred in all, irrespective of whether the solutions were or were not stirred, a procedure claimed to be essential by Hodgkin and Katz for solutions in the higher concentrations.

In all the concentrations of $CaCl_2$ which we used, the axoplasm underwent dispersion within 25 seconds. At 0.68 M the rate of dispersion became maximal so that at this concentration and at 1.36 M and even at 2.72 M the axoplasm dispersed within an average period of 10 seconds.

Micromanipulative Experiments. In the first experiments, our concern during the preparation of the nerve fibers, was to eliminate all possible trace of the Ca of the sea water. For this reason, the intact mantle of the squid was freed of sea water by washing in distilled water. It was then bathed in a calcium-free solution of NaCl and KCl (19:1) isotonic with Woods Hole sea water. A 4–5 inch length of the giant fiber, the average diameter of which is about 500 micra, was then dissected out with the cut ends securely tied with a fine silk thread to occlude the lumen. In the earlier experiments the fibers were kept in the calcium-free solution throughout. In order to reduce trauma, no special effort was made to clean the fiber of its surrounding connective tissue. The isolated nerve was now placed in a shallow groove on a lucite

plate and held in place by the threads attached to each end. In every case it was possible to see clearly the translucent axon through the surrounding tissue. In the later experiments the nerves were dissected in cold, running sea water, a method found by other investigators on the squid nerve to be the most approved way of maintaining the fiber in a normal state.

The micropipettes were of glass tubing drawn in a microflame. Some were long and tapering with diameters 30 micra across at a distance of 600 micra from the microtip. The aperture at the tip measured less than 4 micra across. The pipettes used were either horizontal, in which case they were thrust deep into and along the interior of the fiber from one end where the fiber hung over the edge of the lucite plate. In other cases the pipette was bent in a flame and was thrust either at right angles or diagonally from above into the interior of the axon, without reaching the lower surface. All the injections were done so that the injected oil or solutions lay well within the axoplasm.

The following record of results were found to be similar, irrespective of whether the fibers had been dissected in sea water or in calcium-free salt solutions.

1. *Air.* From an air-filled pipette, it was not possible to inject air in small droplets, since the pressure to overcome the inertia at the outset caused an uncontrollable amount of air to rush out and the injection could not be stopped until the air exuding had formed a relatively large bubble. However, it was possible to introduce small air bubbles by alternating air with oil in the pipette. This was produced by drawing into the micropipette, a fine emulsion of air in oil. Irrespective of their size within visible limits the air bubbles were ovoid, the long axis of the drop being always arranged along the long axis of the axon.

2. *Paraffin oil.* These injections also confirmed previous findings (3) that the interior of the normal, untreated axon is a jelly in which the injected oil drops, no matter how small within visible limits, assume oval shapes always parallel with the long axis of the axon. In the case photographed, Fig. 1, the injection had been continued until the drop reached its maximum cross diameter. With still continued injection the oil column progressively lengthened but with no further widening.

The width of the ovoid drops when first introduced varied with the location where the drop had been deposited. The drops were broad oval in the central region, and became increasingly narrow the closer the drop was to the periphery. In the cortex of the axoplasm the drops assumed narrow slender shapes with rounded conical ends. This indicates a longitudinal ultra-structure throughout the axoplasm, the density of which decreases on passing from the periphery to the central axis of the axon.

On close examination the oil drops could be seen to be coated with a thin, structureless, skin-like layer. This prevented union of adjacent oil drops. In some instances the axoplasm was found to be liquid, in which case the oil drops on injection assumed spherical shapes. This condition was found frequently in isolated nerves which had been left in sea water for over half an hour or longer.

Fig. 1.

Fig. 2.

Fig. 1. Oval shape of oil drop assumed in the jellied axoplasm.
Fig. 2. Spherical shape of same oil drop after axoplasm in vicinity had been injected with $CaCl_2$.

Darkfield photographs of an oil drop which had been injected into the axoplasm of a giant nerve axon the cross diameter of which was 600 micra. The external surface of the axon had not been completely freed of its surrounding fascia. Shaft of micropipette can be seen within the axoplasm to the upper right of drop. The size of the oil drops with reference to the cross diameter of the axon in these photographs is two to three times the size usually microinjected.

3. *Aqueous solutions of pH color indicators.* These were 0.4 per cent solutions of phenol and of brom cresol purple. Micro amounts were injected into the interior of the axon while in its jellied state. The color clearly showed a definite path taken by the injected solution. As long as the tip of the micropipette lay definitely within the axoplasm, the exuding solution streamed to the central axis of the axon, along which it travelled rapidly in both directions longitudinally along the central axis of the axon. Thence the color diffused radially and within several seconds the axon had become evenly colored throughout. Brom cresol purple, whether it had, prior to the injection, the yellow color of its acid or the blue of its alkaline range, turned quickly to the purple blue of the extreme alkaline range of the indicator. With phenol red the color, diffusing throughout the axon, always took on the yellow color of its acid range. The two indicators thus assumed colors consistent with a pH within the range of 6.8 to 7.0. Evidence of an acid of injury was demonstrated by tearing an isolated axon, the jellied interior of which had been

colored blue with a 0.4 per cent aqueous solution of brom cresol purple. Tearing caused the blue color of the axoplasm to change immediately to a persisting yellow along the fringes of the tear.

4. *Water and solutions of electrolytes.* Distilled water could be microinjected in relatively large amounts and no observable change was noted. The water diffused through the jellied axoplasm and along its length without appreciably changing the consistency.

The effect of the solutions of various electrolytes on the physical state of the axoplasm was tested by noting the effect of the injected solutions on the shape of previously injected paraffin oil drops. All the solutions injected were of the chlorides of monovalent and divalent cations in concentrations isotonic with Woods Hole sea water, i.e., 0.52 M for the monovalent and 0.34 M for the divalent salts. When NaCl or KCl was injected the previously introduced ovoid oil drops showed no change in shape, exhibited no motion and, when pushed about, became deformed indicating that the drops lay in a resisting, jettied medium.

When chlorides of the divalent cations, Ca, Sr, Ba or Mg, were injected in concentrations of 0.34 M, there was a striking reaction. In the vicinity of the injection the skin-like coating of the oval oil drops disappeared, the drops rounded into spheres which now exhibited shimmering movements, indicating that they were in a highly fluid medium, Fig. 2. The rate and intensity of the liquefying action of the salts of the divalent cations were found to be in the order: Ca and Sr $>$ Mg $>$ Ba, being almost immediate for Ca and Sr, and delayed several minutes for Mg and still longer for Ba.

Sea water when injected also caused the oil drops at the site of injection to round up indicating that the concentration of the divalent cations in sea water is sufficient to induce liquefaction of the axon material. Sometimes when an isolated length of an axon had been allowed to stand for a long time in sea water, the axoplasm was found to be liquid. On examining this phenomenon more closely it was found that the solating effect was first noticed in those regions adjacent to the branches which had been severed at the time that the fiber had been dissected from the original position in the squid mantle. Evidently the liquid regions were where sea water had seeped in through the cut ends of the branches.

When the relatively slow acting $BaCl_2$ was injected, an interesting detail was noted in the change in shape of the oil drop during the liquefaction of the jellied axoplasm. A fine pipette, filled with 0.34 M $BaCl_2$ was inserted so that its tip lay alongside the middle of a cylindroid oil drop, 240 microns

long and 30 microns across. When the solution was gently ejected, an indentation appeared on that surface of the oil closest to the aperture of the pipette. As the injection was continued, the indentation deepened into a pronounced constriction which cut the drop in two. The droplet beyond the site of injection, rounded completely and floated away. The other droplet maintained its original elongated contour for over 10 minutess. The slowness in the progress of the liquefying action on the axoplasm was indicated by the observed fact that the liquefaction first affected the region just beyond the tip of the pipette, and spread backward along the shaft of the pipette immediately adjacent. This part of the oil drop never rounded completely, indicating that the liquefying action of the solution had become ineffective by too much dilution while spreading from the site of injection.

5. *Sodium alizarin sulfonate.* A test was made to ascertain the effect of introducing a calcium precipitant into an axon previously liquefied by $CaCl_2$. The precipitant used was a 1 per cent, aqueous solution of sodium alizarin sulfonate which had been used in microinjection on the amoeba (8). The alizarin solution is red in color in a medium more alkaline than pH 4.0. When injected into a previously untreated, jellied axoplasm it caused no visible change. However, when the alizarin salt was introduced into the axoplasm which has been previously liquefied by an injection of $CaCl_2$, there occurred instantly a visible precipitation of fine purple crystals. This was immediately followed by a stiffening of the axon material in which the oil drops, previously spherical, now became irregularly distorted in the newly formed jelly. It is significant that the reappearance of the jelly state caused the oil drops, now held in the jelly, to assume irregular shapes with no reference to the original linearly oriented arrangement characteristic of the normal fiber.

The successive effects on the axoplasm of the injection of $CaCl_2$ followed by the injection of a calcium precipitant are shown dramatically in the following experiment: A fiber, freshly prepared in sea water, was given injections successively of oil, $CaCl_2$ (0.3 M) and of Na alizarin sulfonate (1 per cent). A pipette was filled by way of its microtip, first, with a quantity of the alizarin solution, then a short column of oil followed by a solution of the $CaCl_2$ and finally a column of oil which reached to the microtip. The filled pipette was then inserted into the axon and pressure applied to the injection syringe. The first to exude was the oil which assumed the long ovoid shape characteristic of the oil when introduced into the jellied axoplasm of a normal axon. The oil was followed by the solution of $CaCl_2$ which lique-

fied the axoplasm exhibited by the immediate change in shape of the oil drop to that of a sphere. Oil was next extruded and also appeared as spheres. Then followed the alizarin solution, whereupon there occurred a visible precipitation of minute, purple, acicular crystals in the region of the injection. The precipitation spread as the injected solution diffused throughout the axoplasm and the spherical oil drops present became distorted and were now found to be held firmly in a stiff jelly. Similar effects were observed when the sodium alizarin S was injected following the liquefying action of Sr, Mg and Ba.

DISCUSSION AND CONCLUSIONS

It is well known that the axon of the giant nerve fiber of the mantle of the squid is a composite of several small axons each from its ganglionic cell body. The giant axon, which may attain the cross sectional dimensions of 600 microns, appears glass-clear and homogenous and is surrounded by a relatively tough enveloping sheath. The lack of resistance, met with in pushing about a drop of oil injected into the axoplasm indicates the completeness of the mergence of the several axons of the original nerve fibers. The giant axon reacts as a uniformly, gelatinized structure. The existence of a longitudinal orientation is indicated by the long ovoid shape of introduced oil drops. The long axes of these drops are always parallel with the long axis of the axon. It is concluded that the axon material of a fresh nerve possesses a uniform, linearly arranged structure of ultra microscopic dimensions. The firmness of the jelly of the axon is sufficient to affect the shape of a paraffin oil drop but not to prevent the rounding of an injected air bubble. Water injected into the axoplasm diffuses readily, manifesting the aqueous nature of the axoplasmic jelly.

The axoplasm undergoes liquefaction when exposed to salts of divalent cations while the gel state is preserved by salts of monovalent cations. The results place the axoplasm in a special category as differing in this respect from the protoplasm of a great variety of living cells.

The significance of the alizarin experiments, in which the liquefied axoplasm could be reversed to a gel by the precipitation of calcium salts, lies in the fact that the longitudinal orientation of the gel structure can be destroyed. The return to the gelated state does not bring back the linear arrangement of the original gelated axoplasm. This was indicated by the fact that the oil drops, which were spherical in the liquefied axoplasm, now became irregularly distorted instead of reassuming their previously long ovoid shapes.

Protoplasm, except when gelated, is essentially a fluid and filamentous

processes of it when extruded tend to be beaded and break into fluid spheres (2). It is to be recalled that the axon of a nerve fiber is probably the longest extended filamentous process of protoplasm known among living cells. The gelated state of the axoplasm of a normal fiber may be a prime factor in the maintenance of such a filamentous extension.

The property of the axoplasm in being liquefied by the salts of divalent cations places the axoplasm in a category different from that of the material constituting the protoplasm of cells in general. It is known that certain collagenous tissues, e.g., chondroitin of cartilage are not coagulated by $CaCl_2$. The axoplasm possibly consists of a type of protein similar to collagen or the chondroitins in not reacting to calcium chloride the way that the proteins of the interior of living cells generally do.

In our first experiments care was taken when dissecting out the fibers, to eliminate all possible traces of the calcium chloride of the sea water. Later we found that this was not necessary. The fact that the gel state persists in these fibers is good evidence for the relative impermeability of the intact fiber to the calcium of sea water. Conversely, the injection of one tenth the concentration of normal sea water was found to create a pronounced liquefaction of the interior of the axon. This should indicate that very little if any of the calcium penetrates the intact fiber. If, however, a nerve subjected to the trauma of operational procedure is allowed to stand in sea water for some time, the interior of the axon becomes liquid. This is explained by a slow progressive penetration of divalent cations which include calcium through the branches of the nerve cut open during the surgical removal of the nerve.

Hodgkin and Katz, in looking through the literature for findings analogous to theirs on the axon, referred to Heilbrunn's claim that calcium salts in the environment decrease the viscosity of the Stentor, while salts of sodium and potassium do the reverse. We were not able to confirm Heilbrunn's findings and find that the interior of Stentor reacts to the salts in a way similar to the protoplasm of the fresh water amoeba and of other cells with which we have experimented.

REFERENCES

1. Chambers, R., The physical properties of protoplasm. Lecture, Mayo Foundation, Rochester, Minn., 1926.
2. —— *Am. Naturalist.*, **72**, 141 (1938).
3. —— *Biol. Bull.*, **93**, 191 (1947).
4. Chambers, R., and Reznikoff, P., *J. Gen. Physiol.*, **8**, 369 (1926).
5. Heilbrunn, L. V., *Am. J. Physiol.*, **64**, 481 (1923).
6. Hodgkin, A. L., and Katz, B., *J. Exptl. Biol.*, **26**, 292 (1949).
7. Kerr, T., *Protoplasma*, **18**, 420 (1933).
8. Pollack, H., *J. Gen. Physiol.*, **11**, 539 (1928).

CULTIVATION OF OVARIAN GRANULOSA CELLS AFTER COOLING TO VERY LOW TEMPERATURES

A. U. SMITH

National Institute for Medical Research, Mill Hill, London Great Britain

Received September 27, 1951

In recent years much interest has been aroused in the possibility of preserving at very low temperatures living cells and tissues from warm blooded animals. Various materials from birds and mammals have been stored in this way and studied both *in vitro* and *in vivo*. Thus, spermatozoa of the domestic fowl and of several mammalian species when suspended in glycerol-containing media show good revival of motility after cooling to and storage at —79° C for long periods (25, 29, 30). It has been shown, furthermore, that fowl spermatozoa retain their fertilizing power after storage at —79° C or at —196° C, provided that after thawing and prior to insemination the glycerol in the medium is removed slowly by dialysis (24). Several workers have reported growth of chick embryonic tissues when cultured *in vitro* after exposure to very low temperatures (8, 13, 17, 23).

Much work has been done on storage of mammalian tumour cells at very low temperatures and material so stored may give rise to tumours when grafted into animals, and may even grow in tissue cultures (5, 6, 9, 10, 12, 13, 19, 20, 32, 33). Various normal mammalian tissues have also been grafted successfully after low temperature storage. While grafts of bone and blood vessels so stored apparently act only as scaffolding to hasten the growth of the host's tissues (11, 34) the epithelium of mammalian skin and thyroid tissue will proliferate vigorously when thawed and grafted back autoplastically after being frozen to and kept at very low temperatures (4, 7, 18). Some tissue culture studies have also been carried out with frozen mammalian tissues, and Klinke (13) reported that kidney tissue from 6 weeks old rabbits, frozen in liquid nitrogen and subsequently cultured, occasionally yielded growths of typical renal epithelium.

Little work has so far been done on low temperature storage of ovarian tissue from mammals. Payne and Meyer (22) showed that rat ovarian tissue after freezing rapidly in liquid air failed to grow or function when implanted into the anterior chamber of the eye of spayed females. Smith (28)

noted that fertilized rabbit eggs which had been frozen at —79° C failed to segment when cultured *in vitro* after thawing; a few of the surrounding ovarian granulosa cells of the corona radiata and cumulus, however, survived this treatment and grew. A series of experiments was subsequently carried out to study the effects of rate of cooling, temperature of storage and presence of glycerol on the growth *in vitro* of ovarian granulosa cells and the fate of ovarian tissue when grafted back into animals. A preliminary account of this work has been given by Parkes and Smith (21), and the object of this communication is to report in detail the tissue culture experiments.

MATERIAL AND METHODS

Collection of serum. Rabbit blood was obtained from the marginal ear vein of lop-eared does, and the serum separated after clotting.

Preparation of embryo extract. Rabbits were killed on the 18th day of pregnancy. The embryos were chopped finely and suspended in twice their volume of Simm's solution (26). After standing for 2 hrs. at room temperature the suspension was centrifuged at 2500 r.p.m. The supernatant fluid was taken off and stored at —10° C until required for use.

Preparation of glycerol solutions. 15 per cent glycerol in saline was made by mixing 15 volumes of glycerol (A. R.) with 85 volumes of sterile 0.85 per cent sodium chloride. 15 per cent glycerol in serum was prepared by mixing 15 volumes of glycerol with 85 volumes of rabbit serum.

Collection of ovarian cells for cultivation. When tissue from rabbit ovarian follicles is cultured, mixed growths of fibroblasts and epithelial cells are obtained. The corona radiata and cumulus cells surrounding the eggs after ovulation are composed solely of granulosa cells, and when cultured give pure growths of epithelial cells. Unfertilized eggs with their surrounding corona and cumulus were therefore obtained from the fallopian tubes. 42 Dutch rabbits were given an intravenous injection of 50 International Units of chorionic gonadotrophin, and killed by air embolism 13–15 hours later. The fur was moistened with spirits of wine, and the skin incised and dissected from the anterior abdominal wall. The peritoneal cavity was then opened with sterile instruments by a longitudinal incision from the pubic symphysis to the xiphisternum. The ovaries, fallopian tubes and distal half inch of the uterine horns were excised using a second set of sterile instruments and were transferred to sterile petri dishes. The fallopian tubes were cleaned of fat, straightened out and washed through from the uterine end with approximately 2 cc of 0.85 per cent NaCl. The washings were examined under the high power of a binocular dissecting microscope, and the eggs and cumuli transferred to the media in which they were to be cooled or cultivated. All the instruments, solutions, and receptacles used during these manipulations were sterile.

Methods of cooling. 0.5 cc of medium containing the eggs and cumuli was transferred to a sterile test-tube (3″ × 0.3″) with a sterile cotton wool plug or else to an ampoule of similar size which was sealed before cooling. Pieces of rubber tubing 12″

long were attached to the tops of the tubes and ampoules to suspend them in the cooling mixtures. Slow cooling between +20° C and 0° C was carried out by placing the tubes and ampoules into a Dewar flask containing methylated absolute alcohol at +20° C and gradually adding powdered solid CO_2 to lower the temperature at the desired rate. Between 0° C and —79° C the tubes were cooled as for bull spermatozoa (29) in successive stages at —2° C, —5° C, —8° C, —10° C, —13° C, —15° C, —20° C, —30° C, —40° C, —50° C, —60° C and —70° C with one, two, or four mins. at each stage. For rapid cooling to —79° C the tubes and ampoules were plunged directly into Dewar flasks containing freezing mixture at that temperature. Material to be cooled at the maximum rate was drawn up and sealed into the capillary end of a Pasteur pipette, and plunged into liquid air at —196° C. The samples were left for periods varying from one hour to 24 hours at —79° C or at —196° C and subsequently thawed at +40° C.

Technique of cultivation. After freezing and thawing, the eggs in their cumuli were transferred to rabbit serum, and, depending on its size, each cumulus was divided into from two to six pieces. The unfrozen control material was similarly treated. Slide cultures were then set up between slide and coverslip in a minimum quantity of medium (28). A piece of cumulus in a small drop consisting of equal parts of serum and of rabbit embryo extract was mounted on a circular coverslip ($\frac{7}{8}$″ diameter). A similar sized drop, composed of equal parts of serum and Simm's solution (26) was placed on the centre of the slide which was inverted over the coverslip so that the drops met and the fluid spread to the edge of the coverslip. The media all contained 0.02 per cent of streptomycin to ensure bacteriostasis, and it was rare for a culture to be contaminated. The total volume of culture medium in such preparations was approximately 0.01 ml. The size of the drops was judged by eye, so that the amount of fluid was sufficient to spread to the edge of the coverslip without oozing out around the edge, and so that the slide could be inverted without the coverslip slipping off. The coverslip was then sealed in position with a mixture containing 2 parts of paraffin wax (56° C) to one part of vaseline, and the preparations incubated at +37° C and observed at intervals by phase contrast microscopy at +37° C inside a 'Perspex' microscope incubator designed by Mr. J. Smiles and made at the National Institute for Medical Research (27). By this method of cultivation pure growths of ovarian granulosa cells were obtained, and the cells spread in a very thin layer on the slide or coverslip and could be studied individually. Many of the cells began to spread and to send out processes within 1 or 2 hours of cultivation, and cell divisions began to take place within 4 or 5 hours of cultivation.

Freezing of growing cultures under the microscope. Attempts were made to culture ovarian granulosa cells on slides made of 'Perspex' so that they could be frozen under the microscope using the apparatus and technique described by Smith, Polge and Smiles (31). Unfortunately no growth was ever obtained, suggesting that the 'Perspex' was toxic to these cells. A modified method for freezing under the microscope was therefore devised. 3″×1″ microscope slides were cut into three pieces each 1″ square. Aluminium spacing rings 0.5 mm thick and 2 cm in diameter with a 2 mm gap at one side were sealed onto the glass slides, and culture of eggs and cumulus cells in a meniscus drop containing serum, embryo extract and streptomycin, were put up as previously described (28). The preparations were incubated at +37° C in the inverted position, and after 24 hours there was generally a good growth on

the coverslip. A heat-conducting slide holder was made from a copper strip 18″ × 1″ and 0.25 mm thick with a central circular perforation 0.75″ in diameter. A thin strip of cork 6″ × 1″ with a corresponding central circular aperture was attached to the middle of the under surface of the copper strip. Two strips of cork each 2 ½″ × 1″ were attached to the upper surface of the copper strip so that their edges were 1″ apart and the circular aperture was placed midway between them. The copper and cork strips were bound firmly together with adhesive tape.

The slide bearing the growing culture was inserted into this holder so that the central aperture in the copper and cork corresponded with the position of the culture chamber.

A thermocouple was inserted into the culture chamber through the wax seal and the gap in the spacing ring. It was sealed in position with wax, and connected to the circuit of the temperature recording system. The upper and under surfaces of the culture chamber were smeared with a thin film of glycerol, to prevent frosting up. The holder was clamped in position on the microscope stage, and the objective focussed onto the culture. The bare copper strips, which projected 6″ beyond the insulation on either side were bent down at an angle of 135° and dipped into vacuum flasks containing liquid air or alcohol-CO_2 freezing mixture. In this way the preparation was cooled under direct microscopical observation, the rate of cooling being altered by adjusting the temperature of the freezing mixtures, and the length of the conducting arms dipping into them. It was subsequently thawed by dipping the conducting arms into hot water at about +60° C. The slide was then removed from the holder and reincubated at +37° C.

Freeze-drying of ovarian cells. The cumuli were suspended in 0.5 ml serum and transferred to 50 cc round bottomed flasks with B.34 Quickfit necks to fit onto the freeze-drying unit. The flasks were stoppered and cooled slowly to —79° C. They were revolved between —5° and —15° so that the material was frozen in a thin layer. The material was then freeze-dried for 2–5 hrs. It was reconstituted by addition of 0.5 cc of distilled water plus 4.5 cc rabbit serum. The cumuli were then divided up and cultured.

RESULTS

The effect of cooling at different rates in serum. The cumuli from 12 rabbits were suspended in serum. Those from 3 were frozen in liquid air at —196° C, while those from another 3 were frozen at —79° C, and those from 3 more were cooled slowly, taking 40 mins. to reach —79° C. After 2, 4 and 24 hrs. cumuli treated in the different ways were thawed out and cultured. Three rabbits served as controls, and their cumuli were cultured after standing at room temperature for 2, 4 and 24 hrs.

None of the cultures of material frozen in liquid air showed growth at any time, and all the cells looked degenerate (Fig. 1).

In cultures of material frozen at —79° C the majority of cells appeared dead and degenerate, but after 24 hrs. small numbers of isolated spreading cells were found (Fig. 2) and these multiplied over the subsequent 48 hrs. By

contrast, material which had been cooled slowly, taking 40 mins. to reach —79° C, showed, within 2 hrs. of cultivation, 50 to 75 per cent of living spreading cells, and after 24 hrs. vigorous cultures were obtained with small numbers of dead cells scattered among the sheets and clusters of growing ones (Fig. 3). The appearances of these cultures differed little from the control cultures (Fig. 4).

To ensure that these results were not due to differences in the viability of granulosa cells from different animals, another 3 rabbits were killed and their cumuli suspended in serum and divided each into four parts. One portion from each cumulus was frozen at —196° C, a second frozen at —79° C, and a third cooled slowly to —79° C. The remaining portion was left at room temperature. After 2 hrs. they were thawed at +40° C and cultured. The portions frozen at —196° C showed no growth; those frozen at —79° C showed growth of a very few cells, while in those cooled slowly to —79° C at least 50 per cent of the cells were alive and spreading after 4 hrs. of incubation, and gave rise after 24 hrs. to vigorous cultures. In the cultures of unfrozen control material nearly all the cells were sprouting after 4 hrs. incubation, but after 24 hrs. incubation the extent of growth was little greater than in the cultures of cumulus previously cooled slowly.

In other experiments the time taken for slow cooling was varied. It was found that lengthening beyond 15 mins. the time taken to pass from room temperature to 0° C decreased the survival and subsequent growth of cumulus cells. In material which had been cooled from + 20° C to 0° C in 10 mins. there was little difference between cultures of samples which were cooled to —79° C in 2 mins. and 4 mins. stages in each of the 12 freezing mixtures, but they were both distinctly better than those cooled in one min. stages. Cooling very slowly, taking 24 hrs. to pass from 0° C to —79° C gave results inferior to cooling through the same range in 24 mins. Material kept for 2 hrs. and 24 hrs. at —79° C gave the same results.

The effect of glycerol in the suspending fluid, and of slow cooling. The suspending fluids investigated were serum, 0.85 per cent NaCl, 15 per cent glycerol in serum, and 15 per cent glycerol in 0.85 per cent NaCl. In one

---

Fig. 1. Frozen in liquid air (—196° C). ×500.
Fig. 2. Frozen rapidly at —79° C, kept for 20 hrs. at —79° C. ×500.
Fig. 3. Cooled slowly to —79° C, kept for 24 hrs. at —79° C. × 500.
Fig. 4. Unfrozen control. ×500.

The cumulus cells were suspended in serum, frozen by different methods, thawed at +40° C, cultured, and incubated 24 hrs.

The photomicrographs were taken by Phase-contrast microscopy.

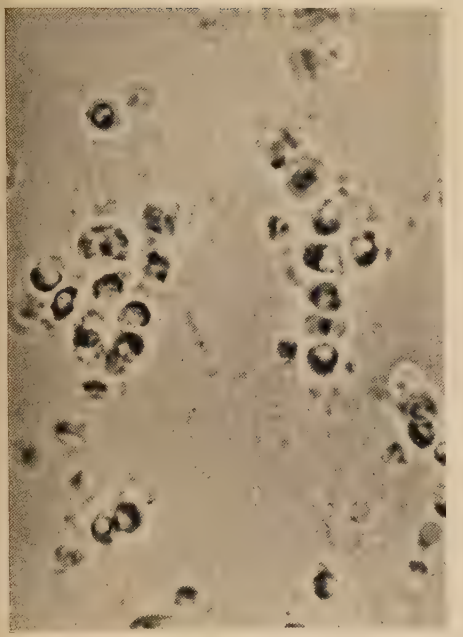


Fig. 1.

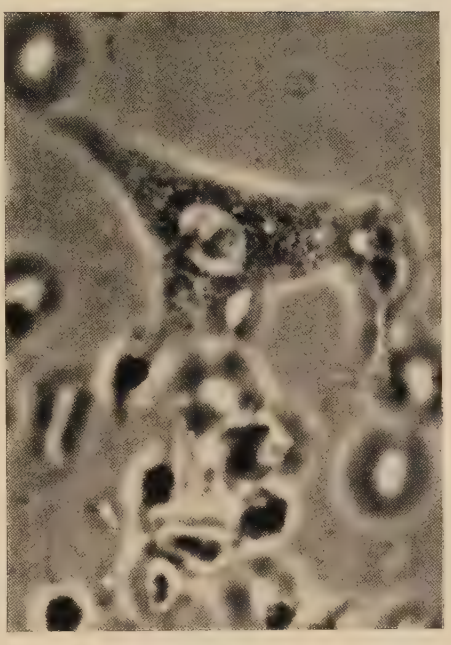


Fig. 2.

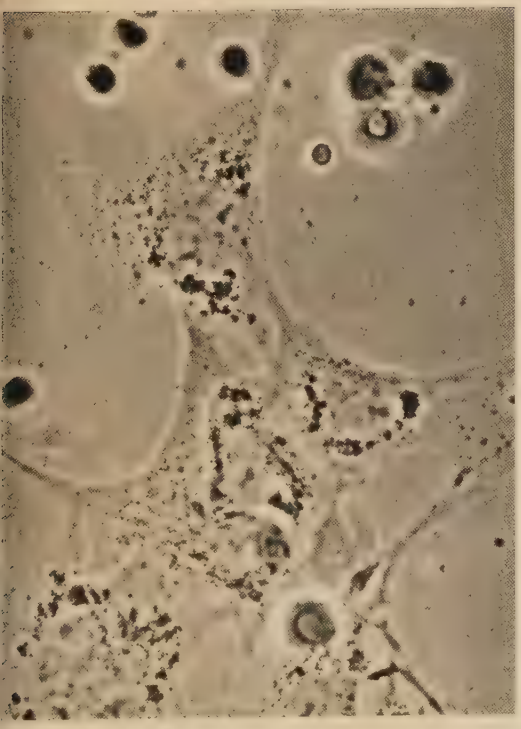


Fig. 3.

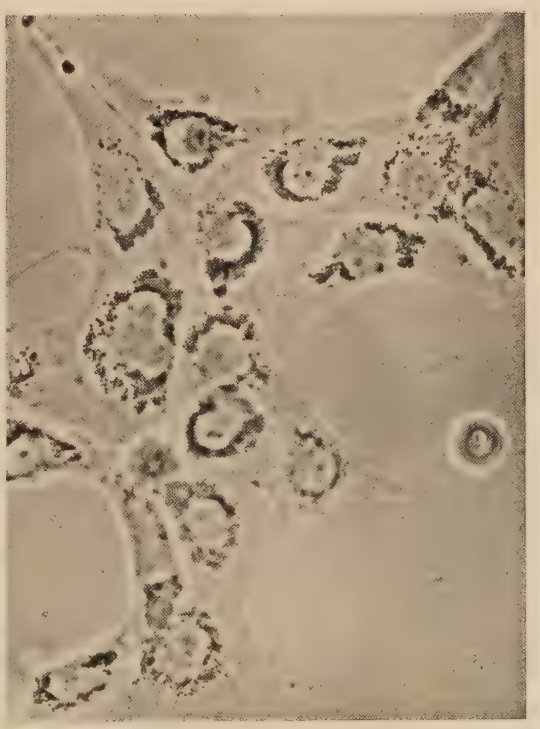


Fig. 4.

experiment the cumuli from each of 4 rabbits were suspended in different media, and half the material was cooled slowly to —79° C and held at that temperature for 3 hrs. while the other half was left at room temperature for 3 hrs. In another experiment the cumuli from three rabbits were divided into eight, and portions from each rabbit suspended in the different media and either cooled slowly or left at room temperature. The material was transferred to fresh serum before cultivation. The results were clear cut. In cultures of unfrozen material which had been soaked either in serum or in 0.85 per cent NaCl, the majority of cells were spreading after 4 hrs. and vigorous growths were obtained after 24 hrs. incubation. Even earlier and better growths were obtained from the cumuli soaked in 15 per cent glycerol in serum. By contrast, in cultures of material which had been soaked in 15 per cent glycerol in 0.85 per cent NaCl only about half the cells were spreading after 4 hrs. incubation, while the remainder appeared in varying degrees disorganised, and after 24 hrs. incubation the growths were inferior to the serum and saline controls and to the glycerol serum group. In cultures of the material suspended and cooled in serum at least 50 per cent of the cells were spreading after 4 hrs. incubation, and very good growths were obtained after 24 hrs., whereas in material suspended and cooled in 0.85 per cent NaCl no spreading cells were seen after 4 hrs. and no growth was obtained after 24 hrs. at +37° C. In cultures of cumulus which had been cooled in 15 per cent glycerol in 0.85 per cent NaCl, on the other hand, about 25 to 50 per cent of the cells were spreading after 4 hrs. at +37° C and after 24 hrs. growths little inferior to the unfrozen controls of this group were obtained. The portions of cumulus which had been suspended and cooled in 15 per cent glycerol in serum showed about 75 per cent of the cells spreading after 4 hrs. incubation and after 24 hrs. incubation the cultures were superior to those of cumulus cooled in serum without glycerol. The effect of rapid cooling to —79° C in presence of 15 per cent glycerol in serum was then tried but only small numbers of isolated cells survived and grew in culture and the results were no better than from rapid cooling in serum without glycerol. Material suspended in 0.85 per cent NaCl and cooled rapidly to —196° C or to —79° C was also cultured, but no living cells were found in any of the preparations.

These results indicate that, in presence of serum, 15 per cent glycerol may have a beneficial effect on the growth *in vitro* of ovarian granulosa cells, and that it enhances the protective action of serum against slow cooling to very low temperatures. Exposure to 15 per cent glycerol in 0.85 per cent NaCl, on the other hand, will inhibit the growth of about 50 per cent of these

cells, but a high proportion of those which withstand its effects at room temperature will also withstand exposure to very low temperatures.

The effect of freezing on growing cultures. Six cultures of cumulus were set up on 1″ square slides using spacing rings as described above. After 24 hrs. they all showed good growths. Four cultures were cooled slowly under direct microscopical observation, taking 10 mins. to pass from + 20° C to 0° C, and 10, 20, 30 and 40 mins. to pass from 0° C to —45° C. Freezing took place between —10° C and —16° C. They were thawed out and re-incubated at +37° C. After a further 24 hrs. incubation, groups of living growing cells were seen in each culture; the majority of cells were, however, degenerate and dead.

Two other cultures were cooled as rapidly as possible by immersing the copper conducting arms in liquid air at —196° C so that freezing took place in 1 ½ mins. and —50° C was reached in 3 mins. In these cultures no cells survived.

Freeze-dried cumulus. The cumuli from 6 rabbits were freeze-dried, then reconstituted and cultured. 35 cultures were made, but although many of the cells were indistinguishable from freshly collected cumulus cells there was no *in vitro* growth.

DISCUSSION

Work on preserving living cells at very low temperatures has, for many years, been dominated by the theory that ice formation must be avoided. Hitherto this has been done either by dehydrating the cells *in vacuo* before exposing them to very low temperatures (1, 2, 3) or else by ultra-rapid cooling and re-warming so that the material passes to and from the vitreous state without intermediate crystallisation (14, 15). Sometimes a combination of partial dehydration and ultrarapid freezing has been used (16, 17). Becquerel's technique is limited to cells able to withstand total removal of water at room temperature. Luyet's method is limited to minute amounts of material.

This theory, however, was severely shaken by the work of Breedis (5) on slow-freezing of leukaemia cells, and more recently by the revival of cells after slow freezing in glycerol-containing media (21, 24, 29, 30). The results reported in this paper show conclusively that follicular granulosa cells from the adult rabbit ovary will grow and multiply *in vitro* after slow but not after rapid cooling to very low temperatures. They demonstrate, furthermore, that cell survival depends on the surrounding medium. In presence of 15 per cent glycerol the cells might be partially dehydrated by

osmotic withdrawal of water before or during freezing, but it is more difficult to explain in this way the protective action of serum. The results have opened up interesting practical possibilities and difficult theoretical problems.

SUMMARY

1. Ovarian granulosa cells from the corona radiata and cumulus surrounding freshly shed rabbit eggs have been cultured after cooling to very low temperatures in different fluids.

2. When suspended in serum and cooled slowly to —79° C, 50 to 70 per cent of the cells survived and grew when cultured. After freezing rapidly at —79° C only a few cells survived, while after freezing rapidly at —196° C none survived.

3. Addition of 15 per cent glycerol to serum improved the survival and subsequent growth after slow but not after rapid cooling to —79° C.

4. Material suspended in 0.85 per cent NaCl and cooled either slowly or rapidly showed no survival or growth of cells when subsequently cultured, whereas when suspended in a solution containing 15 per cent glycerol in 0.85 per cent NaCl, about 25 to 50 per cent of the cells survived slow cooling to —79° C, and grew when cultured.

5. When growing cultures were cooled slowly to —50° C and subsequently thawed, a small proportion of cells survived and resumed growth. After rapid cooling there was no survival.

The photomicrographs were taken by Mr. J. Smiles, National Institute for Medical Research, to whom I am much indebted. I am most grateful to Dr. A. S. Parkes, F.R.S., for his interest in this work.

REFERENCES

1. Becquerel, P., *Compt. Rend. Acad. Sci., Paris*, **148**, 1052 (1909).
2. —— *ibid.*, **202**, 978 (1936).
3. —— *ibid.*, **231**, 1392 (1950).
4. Blumenthal, H. T., and Walsh, L. B., *Proc. Soc. Exptl. Biol.*, **73**, 62 (1950).
5. Breedis, C., *J. Exptl. Med.*, **76**, 221 (1942).
6. Breedis, C., and Furth, J., *Science*, **88**, 531 (1938).
7. Briggs, R., and Jund, L., *Anat. Rec.*, **89**, 75 (1944).
8. Bucciante, L., Proceedings of the 8th International Congress of Refrigeration. (In press.)
9. Craigie, J., *Brit. J. Cancer*, **3**, 268 (1949).
10. —— *Brit. Med. J.*, (**ii**), 1487 (1949).
11. Deterling, R. A., Coleman, C. C., and Parshley, M., *N. Y. Med. J.*, **6**, 19 (1950).
12. Gye, W. E., Begg, A. M., Mann, I., and Craigie, J., *Brit. J. Cancer*, **3**, 259 (1949).
13. Klinke, J., *Growth*, **3**, 169 (1939).

14. Luyet, B. J., in Preservation of the Formed Elements and of the Proteins of the Blood. American Red Cross, 1949.
15. Luyet, B. J., and Gehenio, P. M., Life and Death at Low Temperatures. *Biodynamica.* Normandy, Missouri, 1940.
16. Luyet, B. J., and Gonzales, F., *Federation Proc.*, **9**, 82 (1950).
17. —— *ibid*, **10**, 52 (1951).
18. Mider, G. B., and Morton, J. J., *Amer. J. Cancer*, **35**, 502 (1939).
19. Passey, R. D., and Dmochowsky, L., *Brit. Med. J.*, (**ii**), 1129 (1950).
20. Passey, R. D., Dmochowsky, L., Lasnitzky, I., and Millard, A., *Brit. Med. J.*, (**ii**), 1134 (1950).
21. Parkes, A. S., and Smith, A. U., *J. Endocrinol.*, **7**, xxxix (1951).
22. Payne, M. A., and Meyer, R. K., *Proc. Soc. Exptl. Biol.*, **51**, 188 (1942).
23. Pierangeli, G., Proceedings of the 8th International Congress of Refrigeration. (In press.)
24. Polge, C., *Nature*, **167**, 949 (1951).
25. Polge, C., Smith, A. U., and Parkes, A. S., *ibid.*, **164**, 666 (1949).
26. Sanders, H. S., and Simms, M., *Arch. Path.*, **33**, 619 (1942).
27. Smiles, J. (In press.)
28. Smith, A. U., *Nature*, **164**, 1136 (1949).
29. Smith, A. U., and Polge, C., *Vet. Rec.*, **62**, 115 (1950 a).
30. —— *Nature*, **166**, 668 (1950 b).
31. Smith, A. U., Polge, C., and Smiles, J., *J. Roy. Microscop. Soc.*, **71**, 186 (1951).
32. Snell, G. D., and Cloudman, A. M., *Cancer Res.*, **3**, 396 (1943).
33. Warner, P. T. J. C. P., Gostling, J. V. T., and Thackray, A. C., *Brit. J. Cancer*, **4**, 396 (1951).
34. Wilson, P. D., *Brit. Med. J.*, (**i**), 1257, May 27th (1950).

DIE WIRKUNG VON ADENOSINTRIPHOSPHAT, MUSKEL-ADENYLSÄURE, HEFEADENYLSÄURE UND ADENOSIN AUF DIE TEILUNG DES SEEIGELEIS BEI ANWENDUNG VON HEMMUNGSFAKTOREN

GEORG KRISZAT

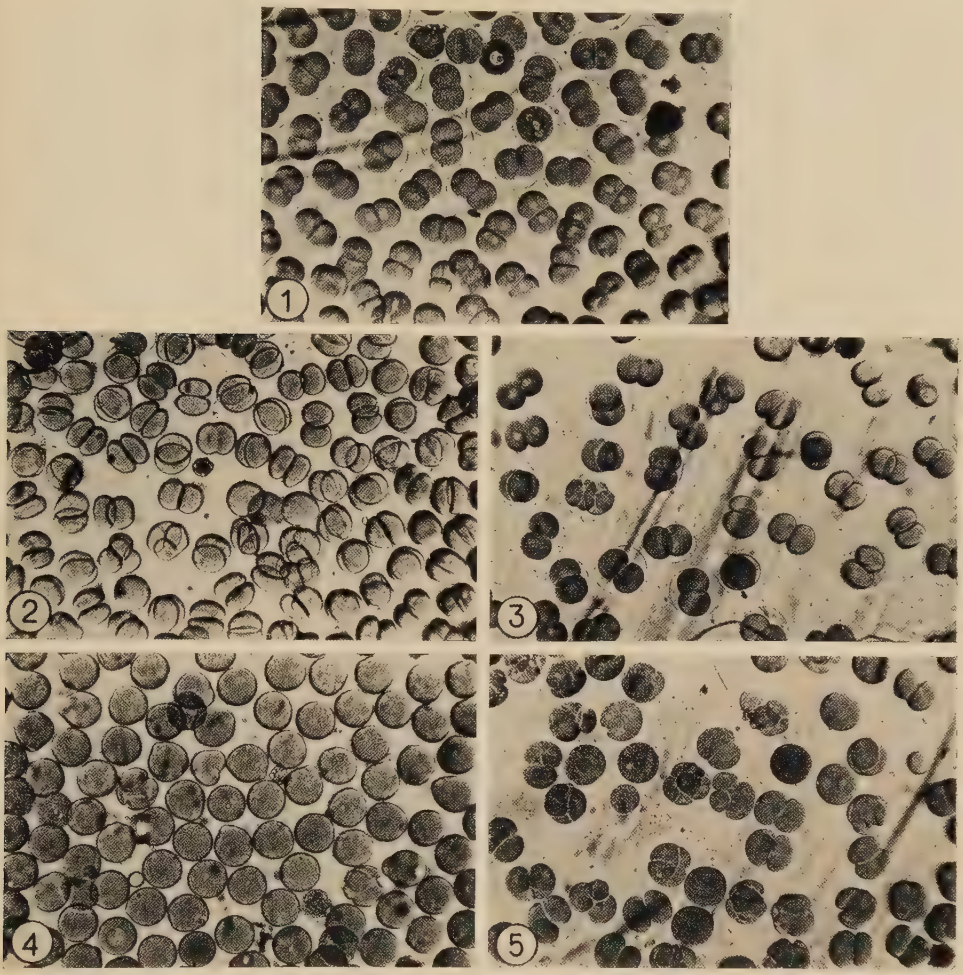
Wenner-Grens Institut für experimentelle Biologie, Stockholm, Schweden

Eingegangen am 25. Oktober, 1951

Runnström und Kriszat (8) konnten nachweisen dass ATP einen deutlichen Effekt auf das Zytoplasma des Seeigeleis hat, und dass bei ATP-Behandlung sich die Rigidität des Plasmas erhöht. In früheren Arbeiten von Runnström (7) und Wicklund (10) ist gezeigt worden dass ATP einen Einfluss auf die Befruchtung des Seeigeleis hat und dass es Veränderungen des Plasmas hervorruft, die sich sowohl bei der Befruchtung als auch bei der Teilung auswirken. Runnström und Kriszat (9) konnten gleichfalls nachweisen dass ATP die Teilungstendenz der Eier verbessert so dass Eier die vor der Befruchtung mit ATP behandelt worden waren, sich besser teilten als solche in unbehandelten Kontrollen, in denen aus bestimmten Gründen der Zustand der Eier nicht vollständig normal war, so dass die Teilung unter der sonst beobachteten Quote lag. Diese Erscheinung zeigte sich noch deutlicher bei Anwendung von Hemmungsfaktoren (3), die eine Teilung der Eier ganz oder teilweise hindern, wenn hemmend wirkende Lösungen in entsprechender Konzentration angewendet werden. In dieser Weise wirkten auch Heparin oder in Lösung gebrachte Gallerthüllensubstanz (9). In diesem Fall war die Wirkung des ATP derart dass die Hemmungsfaktoren ganz oder teilweise aufgehoben wurden, je nachdem in welcher Konzentration die Lösungen angewendet worden waren. ATP-Zusatz verbesserte in diesen Fällen nicht nur die Teilungsquote sondern bewirkte im Allgemeinen eine regelmässigere Teilung der Eier, die sonst unter der direkten Einwirkung des Heparins oder der verschiedenen Lösungen der Gallerthüllensubstanz, teilweise Abweichungen von der normalen Teilung zeigten.

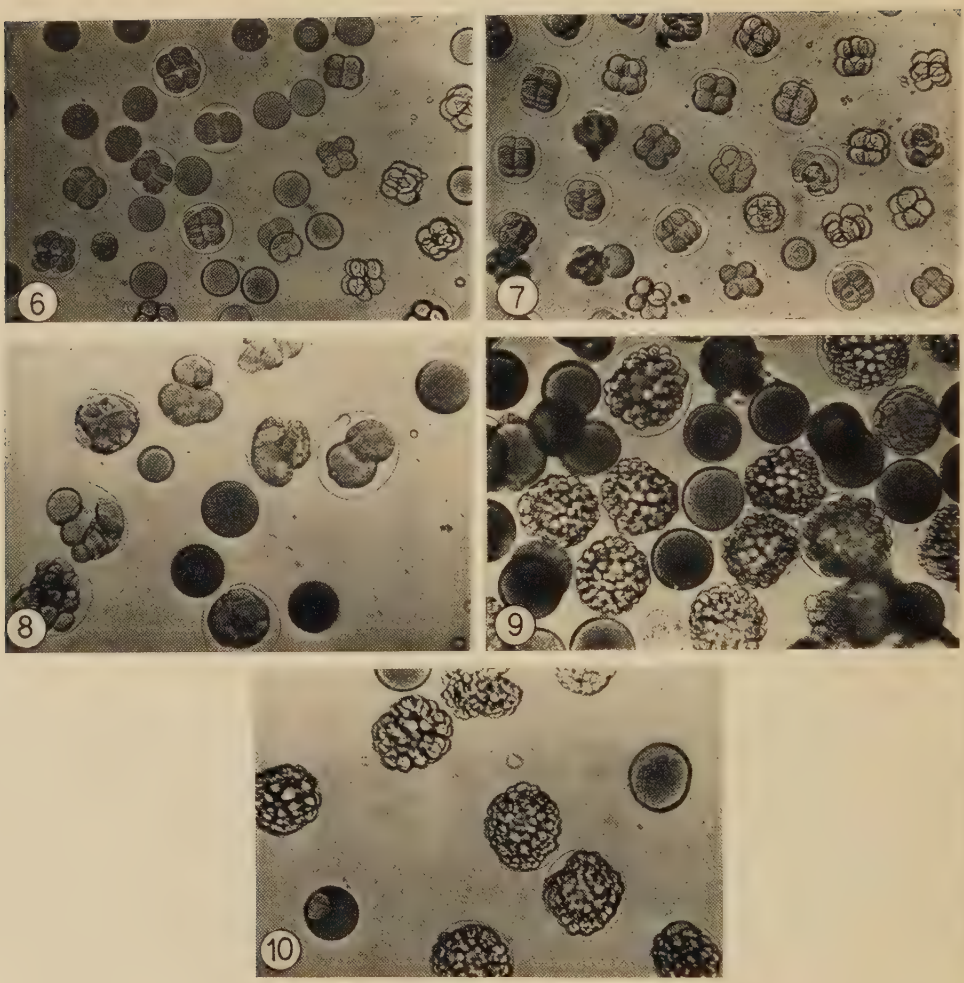
Im Zusammenhang mit den oben erwähnten Befunden war es von Interesse die Wirkung von ATP auf die Teilung eingehender zu studieren.

Es ist möglich dass zu dieser ATP-Wirkung auf das undifferenzierte Plasma eine Parallelle gezogen werden kann zu der Function die ATP in der glatten



Eier von *Psammechinus miliaris* die eine Stunde mit und ohne 0,005 M ATP in hypotonischem Seewasser von 22,5 und 17,5 pro mille vorbehandelt wurden. Danach erfolgte Übertragung in normales Seewasser und Befruchtung. Abb. 1–5.

Abb. 1. Unbehandelte Kontrolle in normalem Seewasser.
Abb. 2. Mit hypotonischem Seewasser von 22,5 pro mille vorbehandelte Eier.
Abb. 3. Mit hypotonischem Seewasser von 22,5 pro mille und 0,005 M ATP vorbehandelte Eier.
Abb. 4. Mit hypotonischem Seewasser von 17,5 pro mille vorbehandelte Eier.
Abb. 5. Mit hypotonischem Seewasser von 17,5 pro mille und 0,005 M ATP vorbehandelte Eier.



Eier von *Psammechinus miliaris* die eine Stunde mit und ohne 0,005 M ATP in hypertonischem Seewasser von 30 pro mille vorbehandelt wurden. Danach erfolgte Übertragung in normales Seewasser und Befruchtung. Abb. 6–7.

Abb. 6. Mit hypertonischem Seewasser von 30 pro mille vorbehandelte Eier.
Abb. 7. Mit hypertonischem Seewasser von 30 pro mille und 0,005 M ATP vorbehandelte Eier.

Eier von *Strongylocentrotus droebachiensis* in normalem Seewasser befruchtet und danach in hypertonischen Seewasser von 40 pro mille mit und ohne Zusatz von 0,005 M Muskeladenylsäure oder Adenosin. Abb. 8–10.

Abb. 8. Hypertonisches Seewasser von 40 pro mille.
Abb. 9. Hypertonisches Seewasser von 40 pro mille und 0,005 M Muskeladenylsäure.
Abb. 10. Hypertonisches Seewasser von 40 pro mille und 0,005 M Adenosin.

und gestreiften Muskulatur bei kontraktilen Vorgängen hat. In diesem Zusammenhange wurde die Frage der Phosphorylierung näher untersucht.

Zur Ergänzung der Versuche mit ATP wurde geprüft ob auch andere Nucleotide wie z. B. Muskeladenylsäure und Hefeadenylsäure oder Nucleoside wie Adenosin unter denselben experimentellen Bedingungen eine Wirkung auf die Teilung haben können.

MATERIAL UND METHODE

Für die Versuche wurden Eier von verschiedenen Seeigelarten verwendet. Die Versuche wurden im März mit den Eiern von *Strongylocentrotus droebachiensis* und im April und Mai mit denen von *Echinus esculentus* durchgeführt. Für den grösseren Teil der Versuche im Sommer wurden Eier von *Psammechinus miliaris* und *Echinocardium cordatum* benutzt. Alle Versuche erfolgten an der zoologischen Station Kristineberg an der schwedischen Westküste. Die Eier von *Psammechinus miliaris* wurden in einem Seewasser von ca. 25 pro mille gehalten. Bei *Echinus esc.*, *Echinocardium cord.* und *Strongylocentrotus droeb.* wurde Seewasser von ca 30 pro mille benutzt. Die Temperatur wurde in allen Versuchen, durch fliessendes Seewasser in dem die Versuchsschalen standen, möglichst konstant gehalten und der jahreszeitlichen Aussentemperatur des Seewassers angepasst. Die Lösungen mit Zusätzen wurden auf pH 8,1–8,2 justiert. Das hypotonische Seewasser wurde durch Verdünnen des gewöhnlichen Seewassers von 25 pro mille durch Zusatz von 2 × im Glasapparat destilliertem Wasser hergestellt. Die Verdünnung erfolgte in der Weise das zu 9, 8, 7, 6, oder 5 Teilen Seewasser jeweils 1, 2, 3, 4 und 5 Teile Aqua dest, zugesetzt wurde, so dass die Verdünnungsstufen 9:1 (22,5 ‰), 8:2 (20 ‰), 7:3 (17,5 ‰), 6:4 (15 ‰) und 5:5 (12,5 ‰) entstanden. Die in den Klammern stehenden Ziffern geben den Salzgehalt dieser Stufe pro mille an. Für die Versuche mit hypertonischem Seewasser wurde normales Seewasser von 25 pro mille langsam eingedampft bis es einen Salzgehalt von 50 pro mille hatte. Dieses Seewasser wurde dann im Verhältnis 9:1 (45 ‰), 8:2 (40 ‰), 7:3 (35 ‰) und 6:4 (30 ‰) mit Aqua dest. verdünnt. In den Tabellen dieser Arbeit ist der jeweilige Salzgehalt pro mille statt der Proportion der Verdünnung angeführt.

Für die Versuche mit 2,4 Dinitrophenol und 4,6 Dinitro-o-kresol wurde normales Seewasser verwendet dem DNP oder DNK in der Konzentration von $4–6 \cdot 10^{-5}$ M zugesetzt war.

Das für die Versuche verwendete Na_4ATP wurde aus Ba_2ATP der Sigma Chemical Co. hergestellt (Reinheit ca. 90 %). Das Na_4ATP wurde durch Na_2SO_4 aus dem Ba_2ATP ausgefällt, durch Zentrifugieren von dem unlöslichen Rückstand gereinigt und auf 0,1 M gebracht.

Das Na_4ATP wurde in allen Versuchen in einer Konzentration von 0,005 M zugesetzt. Infolge das Ca Gehalts des Seewassers kann eine schwache Ausfällung durch das ATP erfolgen. Sie liegt nach früheren Berechnungen innerhalb geringer %-Mengen und verringert sowohl die Gesamtkonzentration des ATP als auch den Ca-Gehalt des Seewassers nicht wesentlich (8). Die für die Versuche verwendete Muskeladenylsäure war gleichfalls ein Präparat der Sigma Chemical Co. Die Hefeadenylsäure wurde

von F. Hoffman La Roche und Co. bezogen und das Adenosin von der Kemikaliebolaget, Stockholm. Alle drei Verbindungen kamen in der Konzentration von 0,005 M zur Anwendung. Für die Versuche wurden jedes Mal frische auf pH 8,2 justierte Lösungen im Seewasser hergestellt.

In den meisten Versuchen ist in der Weise vorgegangen worden dass die Eier zunächst in normalem Seewasser befruchtet wurden. War die Befruchtung einwandfrei, so wurden die Eier unmittelbar danach in die verschiedenen Lösungen übertragen.

In einer Anzahl von Versuchen ist aber auch die Einwirkung von ATP vor der Befruchtung in hypo- und hypertonischen Lösungen geprüft worden.

Bei den Versuchen bei denen ATP vor der Befruchtung zugesetzt wurde, kamen die Eier zunächst für eine Stunde in die verschiedenen hypo- oder hypertonischen Lösungen mit und ohne ATP nebst einer unbehandelten Kontrolle in Seewasser. Nach entsprechender Einwirkung der Lösungen wurden die Eier in normales Seewasser überführt und dort befruchtet. Dabei wurde die Abhebung der Befruchtungsmembran und die Anzahl der befruchteten Eier zu jedem Parallelversuch verglichen.

In einer weiteren Versuchsserie mit hypo- und hypertonischem Seewasser wurde ATP in gleichen Zeitabständen nach der Befruchtung zugesetzt. In diesen Versuchen wurden die Eier des gleichen Materials zunächst alle in normalem Seewasser befruchtet und danach in die verschiedenen hypo- oder hypertonischen Lösungen übertragen. Zu jeder Versuchsserie wurde dann in sich wiederholenden Abständen von je 10 min., ATP zugesetzt. Daneben befanden sich gleichzeitig Kontrollen der gleichen Verdünnungsstufen ohne ATP-Zusatz.

Bei den Versuchen mit Dinitrophenol und Dinitrokresol wurden die Eier zunächst in normalem Seewasser befruchtet und dann in Seewasserserien übertragen, die verschiedene Mengen DNP oder DNK mit und ohne Zusatz von ATP, Muskeladenylsäure, Hefeadenylsäure oder Adenosin enthielten.

Auch bei den Versuchen mit hypertonischem Seewasser mit und ohne Zusatz von Muskeladenylsäure, Hefeadenylsäure oder Adenosin wurden die Eier zunächst in normalem Seewasser befruchtet und dann in die erwähnten Lösungen übergeführt.

In allen Versuchen wurde der Verlauf der Teilung und die Anzahl der geteilten Eier immer zum gleichen Zeitpunkt ausgewertet. Bei *Echinus* und *Strongylocentrotus* erfolgte diese Auswertung zuerst nach 4 Stunden und bei *Psammechinus* und *Echinocardium*, 2 Stunden nach der Befruchtung. Die Auswertung erfolgte durch Auszählung der geteilten und ungeteilten Eier innerhalb der gleichen Fläche. Das Zahlenmaterial wurde für jeden Einzelversuch aus einer Anzahl von 200–300 Eiern gewonnen.

Die in den Tabellen angeführten %-Zahlen sind die Mittelwerte aus der jeweils angegebenen Anzahl von Versuchen. Für eine Reihe von Versuchen ist berechnet worden wie weit die einzelnen %-Zahlen signifikant sind wenn man die Resultate der Serien mit und ohne ATP vergleicht. Signifikante Werte ergaben sich für die Lösungen mit stark hypo- bzw. hypertonischen Seewasser. Die Unterschiede der Teilungsquoten in den einzelnen Lösungen kommen meist in dem Zahlenmaterial ausreichend zum Ausdruck. In einer Reihe von Serien sind die quantitativen Unterschiede nicht signifikant. In diesen Fällen zeigen sich aber sehr oft wesentliche qualitative Unterschiede

im Teilungszustand der Eier mit und ohne ATP. Die Eier weisen in den Serien mit ATP einen regelmässigeren und manchmal auch weitergehenden Teilungszustand auf. Dieses geht auch aus einigen zum Abdruck gebrachten Abbildungen hervor.

ERGEBNISSE

Versuche mit hypotonischem und hypertonischem Seewasser und ATP-Zusatz vor der Befruchtung. Für die Versuche mit hypotonischem Seewasser wurde normales Seewasser im Verhältnis 9:1 (22,5 ‰), 8:2 (20 ‰), 7:3 (17,5 ‰), 6:4 (15 ‰) und 5:5 (12,5 ‰) mit Aqua dest, verdünnt. Jede Verdünnungsstufe wurde mit und ohne ATP angesetzt. Zu jedem Versuch gehörte ausserdem eine Kontrolle in normalem Seewasser. Die Eier von *Psammechinus* oder *Echinus* kamen für eine Stunde in diese Lösungen, danach erfolgte die Überführung in normales Seewasser mit anschliessender Befruchtung. Nach bestimmter Zeit wurde die Anzahl der geteilten Eier ausgewertet. Es zeigte sich in allen Verdünnungsstufen dass ATP-Behandlung vor der Befruchtung eine höhere Teilungsquote und im Allgemeinen auch einen regelmässigeren Teilungszustand der Eier bewirkt. Das wird besonderes in den Lösungen deutlich die sowohl in der Hypotonie als auch in der Hypertonie vom normalen Seewasser stärker abweichen. Die Abbildungen (1–5) zu einem solchen Versuch zeigen eine grössere Anzahl von geteilten Eiern und auch einen regelmässigeren Teilungszustand der Eier, die sich in den Lösungen befinden die ATP enthielten. Beim Vergleich der Mittelwerte aus 4 Versuchen mit *Echinus* in Tabelle I a zeigt sich gleichfalls eine höhere Anzahl von geteilten Eiern in den ATP-Serien und das deutliche Hervortreten der Wirkung des ATP bei weitergehender Hypotonie.

Das gleiche Ergebnis geht aus den Versuchen mit *Psammechinus* hervor. In diesem Falle wurden weiter verdünnte hypotonische Lösungen verwendet und zwar in den Stufen 7:3 (17,5 ‰), 6:4 (15 ‰) und 5:5 (12,5 ‰). Tabelle I b zeigt die Mittelwerte aus 7 Versuchen mit *Psammechinus* in hypotonischen Lösungen.

Bei Behandlung der Eier mit hypertonischem Seewasser vor der Befruchtung zeigten sich ähnliche Ergebnisse wie in den vorher beschriebenen Versuchen mit hypotonischen Lösungen. Diese hypertonischen Lösungen wirkten eine Stunde mit und ohne ATP auf die Eier ein. Dann wurden die Eier in normales Seewasser gebracht und dort befruchtet. Tabelle II a gibt eine Übersicht der Mittelwerte aus 4 Versuchen mit *Psammechinus*. Die Abbildungen 6 und 7 zeigen die Teilungen in der Verdünnung 6:4 (30 p.m.) in einem Einzelversuch.

Hypotonie und ATP vor der Befruchtung.

Die Eier wurden eine Stunde in hypotonischen Lösungen mit und ohne ATP (0,005 M) vorbehandelt und dann in normales Seewasser übertragen und befruchtet.

Tab. I a.

Echinus esculentus.

Seewasser-Lösung ‰	22,5	20	17,5
Zellteilung in % ohne ATP	95,0	67,5	8,7
Zellteilung in % mit 0,005 M ATP	97,5	93,7	68,7

Tab. I b.

Psammechinus miliaris.

Seewasser-Lösung ‰	17,5	15	12,5
Zellteilung in % ohne ATP	72,8	37,2	7,2
Zellteilung in % mit 0,005 M ATP	87,1	51,4	22,8

Hypertonie und ATP vor der Befruchtung.

Die Eier wurden eine Stunde in hypertonischen Lösungen mit und ohne Zusatz von ATP vorbehandelt und dann in normales Seewasser übertragen und dort befruchtet.

Tab. II a.

Psammechinus miliaris.

Seewasser-Lösung ‰	40	35	30
Zellteilung in % ohne ATP	43,3	63,3	81,6
Zellteilung in % mit 0,005 M ATP	61,7	90,0	96,6

Versuche mit hypotonischem und hypertonischem Seewasser und ATP in verschiedenen Zeitabständen nach der Befruchtung. Die vorhergehenden Versuche mit hypotonischem und hypertonischem Seewasser haben gezeigt dass sich die Anzahl der geteilten Eier erhöht, wenn man sie vor der Befruchtung mit ATP behandelt hat. Es war nun von Interesse zu untersuchen wie sich der Zusatz von ATP nach der Befruchtung auswirkt. Gleichzeitig sollte untersucht werden, wie der Zusatz von ATP zu verschiedenen Zeiten nach der Befruchtung auf die einzelnen Teilungsstadien des Eies wirkt. Zu diesem Zweck wurde ATP in gleichen Abständen von je 10 min. bis zu dem Zeitpunkt von 50 min. nach der Befruchtung zugesetzt. Alle Eier wurden zunächst in normalem Seewasser befruchtet. Wenn die Membranbildung gut und die Befruchtung 100 %ig war, so wurden sie in die einzelnen Lösungen übertragen. ATP wurde dann im Abstand von 10 min. zugesetzt und die Anzahl der geteilten Eier in den Lösungen mit und ohne Zusatz nach bestimmter Zeit kontrolliert.

Bei den Versuchen mit hypotonischem Seewasser verursachte der Zusatz von ATP in verschiedenen Zeitabständen nach der Befruchtung keine vermehrte oder regelmässigere Teilung der Eier im Vergleich mit den Lösungen

Hypotonie und ATP nach der Befruchtung.

Die Eier wurden in normalem Seewasser befruchtet und unmittelbar nach der Befruchtung in die einzelnen hypotonischen Lösungen übertragen. Danach wurde jede Serie der hypotonischen Lösungen in Abständen von je 10 min. mit 0,005 M ATP behandelt. Daneben befand sich je eine Kontrolle ohne ATP-Zusatz.

Tab. III a.

Psammechinus miliaris.

Seewasser-Lösung ‰	17,5	15	12,5
	Zellteilung in %		
Kontrolle ohne ATP	41,2	0	0
0,005 M ATP 10 min nach der Befruchtung	39,8	1	0
ATP 20 min. nach der Befruchtung	32,1	0	0
ATP 30 min. nach der Befruchtung	29,2	0	0
ATP 40 min. nach der Befruchtung	21,5	0	0
ATP 50 min. nach der Befruchtung	30,5	0,8	0

ohne ATP. Die Teilungsquoten lagen im Vergleich zu den nicht mit ATP behandelten Eiern annähernd gleich oder etwas niedriger wie aus der Tabelle III a hervorgeht, in der die Mittelwerte aus drei Versuchen mit *Psammechinus* zusammengestellt sind.

Bei den Versuchen mit hypertonischem Seewasser zeigte sich ein Unterschied zwischen den mit ATP behandelten und den nicht behandelten Eiern. Tabelle IV a gibt die Mittelwerte aus 5 Versuchen mit *Psammechinus* und Tabelle IV b aus 3 Versuchen mit *Echinocardium* wieder. In allen ATP Reihen liegen die Werte höher. Weiter geht aus den Versuchen hervor dass die Teilungsquote geringer wird, wenn man ATP nach ca. 30 min. und später zusetzt. Da die Auflösung der Kernmembran bei *Psammechinus* ca. 25–30 min. nach der Befruchtung erfolgt könnte der Rückgang der Teilungsquote nach diesem Zeitpunkt mit diesem Vorgang zusammenhängen.

Versuche mit hypotonischem und hypertonischem Seewasser und Muskeladenylsäure, Hefeadenylsäure und Adenosin nach der Befruchtung. Die Versuche mit den obigen Verbindungen wurde in ähnlicher Weise durchgeführt wie mit ATP. Die Eier wurden vorher in normalem Seewasser befruchtet und dann in die Lösungen von verschiedenem hypotonischem oder hypertonischem Seewasser mit und ohne Zusatz einer 0,005 M Menge von Muskel- oder Hefeadenylsäure bzw. Adenosin übertragen.

Tabelle V a und die Abbildungen 8–10 zeigen das Ergebnis solcher Versuchsserien mit den Eiern von *Strongylocentrotus* in verschiedenen Stufen

Hypertonie und ATP nach der Befruchtung.

Die Eier wurden in normalem Seewasser befruchtet und dann gleich nach der Befruchtung in die einzelnen hypertonischen Lösungen übertragen. Danach wurde zu jeder Serie der hypertonischen Lösungen 0,005 M ATP, in Abständen von je 10 min. zugesetzt. Daneben befand sich je eine Kontrolle ohne ATP-Zusatz.

Tab. IV a.

Psammechinus miliaris.

Seewasser-Lösung ‰	40	35	30
	Zellteilung in %		
Kontrolle ohne ATP	1,3	42,5	80,0
0,005 M ATP 10 min. nach der Befruchtung	35,0	72,5	92,5
ATP 20 min. nach der Befruchtung	36,0	70,0	90,0
ATP 30 min. nach der Befruchtung	32,5	71,2	88,7
ATE 40 min. nach der Befruchtung	17,5	62,5	91,2
ATP 50 min. nach der Befruchtung	12,5	53,7	87,5

Tab. IV b.

Echinocardium cordatum.

Seewasser-Lösung ‰	40	35	30
	Zellteilung in %		
Kontrolle ohne ATP	5,0	73,3	88,3
0,005 M ATP 10 min. nach der Befruchtung	55,0	83,3	95,0
ATP 20 min. nach der Befruchtung	51,6	88,3	93,3
ATP 30 min. nach der Befruchtung	53,3	90,0	95,3
ATP 40 min. nach der Befruchtung	36,6	81,6	94,7
ATP 50 min. nach der Befruchtung	33,6	85,0	94,0

Hypertonie und Muskeladenylsäure, Hefeadenylsäure oder Adenosin nach der Befruchtung.

Die Eier wurden zuerst in normalem Seewasser befruchtet und nach der Befruchtung auf verschiedene hypertonische Lösungen mit und ohne Zusatz der obigen Verbindungen übertragen.

Tab. V a.

Strongylocentrotus droebachiensis.

Seewasser-Lösung ‰	40	35	30
	Zellteilung in %		
Kontrolle ohne Zusatz	12	70	85
0,005 M Muskeladenylsäure	52	82	90
0,005 M Hefeadenylsäure ..	30	68	88
0,005 M Adenosin	55	86	92

Tab. V b.

Echinus esculentus.

Seewasser-Lösung ‰	40	35	30
	Zellteilung in %		
Kontrolle ohne Zusatz	16	78	91
0,005 M Muskeladenylsäure	61	91	98
0,005 M Hefeadenylsäure ..	58	81	95
0,005 M Adenosin	63	85	93

hypertonischen Seewassers mit und ohne Zusatz der obigen Verbindungen. In Tabelle V b ist dergleiche Versuch mit *Echinus* und in Tabelle V c mit *Psammechinus* wiedergegeben. Es handelt sich dabei um Mittelwerte aus je 3 Versuchen.

Tab. V c.

Psammechinus miliaris.

Seewasser-Lösung ‰	40	35	30
	Zellteilung in %		
Kontrolle ohne Zusatz	5	61	89
0,005 M Muskeladenylsäure	50	90	95
0,005 M Hefeadenylsäure	36	79	92
0,005 M Adenosin	59	90	96

Bei Versuchen mit hypertonischem Seewasser lagen die Teilungsquoten in den Serien mit Zusätzen von Muskeladenylsäure, Hefeadenylsäure und Adenosin höher als in den Serien ohne Zusätze. Wesentliche Unterschiede in der Wirksamkeit dieser drei Verbindungen konnten in diesen Versuchen nicht ermittelt werden. Es schien dass Hefeadenylsäure eine etwas schwächere Wirkung zeigte als Muskeladenylsäure.

Die Versuche in hypotonischem Seewasser nach der Befruchtung zeigten keine Wirkung der drei Substanzen und glichen in ihrem Resultat den Ergebnissen mit ATP unter den gleichen Bedingungen. Die Teilungsquoten in hypotonischen Lösungen zeigten keine wesentlichen Unterschiede in den Lösungen mit und ohne Zusatz von M- und H-Adenylsäure oder Adenosin.

Ergänzend muss noch bemerkt werden dass Adenosin und in geringerem Umfange auch Muskel- und Hefeadenylsäure, sowie unter bestimmten Bedingungen auch ATP einen hemmenden Einfluss auf die Entwicklung haben können. Es ist beobachtet worden dass Adenosin vom frühen Blastulastadium ab eine unregelmässige Entwicklung bewirken kann. Es können in solchem Falle grosszellige Formen entstehen in denen die einzelnen Zellen eine Tendenz zum Auseinanderweichen zeigen. Abweichungen von der normalen Entwicklung können auch unter bestimmten Verhältnissen bei den Adenylsäuren und bei ATP beobachtet werden, wenn die Konzentrationen über 0,005 M hinausgehen oder unter gewissen Bedingungen die Lösungen zu lange einwirken.

Bei den ersten Teilungen, auf die sich die vorliegende Arbeit bezieht ist ein solcher Einfluss der betreffenden Verbindungen nicht beobachtet worden.

Versuche mit Dinitrophenol und Dinitrokresol sowie ATP, Muskeladenylsäure, Hefeadenylsäure und Adenosin. Die Wirkungen von 2,4 Dinitrophenol und anderen nitrierten Phenolen und Kresolen auf den Stoffwechsel und die

Dinitrophenol und ATP nach der Befruchtung.

Die Eier wurden zuerst in normalem Seewasser befruchtet und dann in verschiedene Lösungen von Dinitrophenol mit und ohne ATP-Zusatz überführt.

Tab. VI a.

Psammechinus miliaris.

	$6 \cdot 10^{-5}$ M DNP in Seew.	$5 \cdot 10^{-5}$ M DNP in Seew.	$4 \cdot 10^{-5}$ M DNP in Seew.
Zellteilung in % ohne ATP ...	19,0	36,8	48,6
Zellteilung in % mit ATP	30,0	49,3	80,6

Tab. VI b.

Psammechinus miliaris.

	$6 \cdot 10^{-5}$ M DNP in Seew.	$5 \cdot 10^{-5}$ M DNP in Seew.	$4 \cdot 10^{-5}$ M DNP in Seew.
Zellteilung in % ohne ATP ...	22,4	43,8	64,9
Zellteilung in % mit ATP	41,5	61,1	85,4

Teilung der Zelle sind von früheren Autoren eingehend beschrieben worden (1, 2, 4, 5, 6). Im Hinblick auf die spezifische Wirkung von DNP und ähnlichen Verbindungen auf die Phosphorylierung, erschien es wichtig zu prüfen wie weit die hier verwendeten Verbindungen die durch DNP und DNK verursachten Wirkungen wieder aufheben können.

DNP oder DNK wurden in den vorliegenden Versuchen dem Seewasser in drei verschiedenen Konzentrationen zugesetzt. Gleichzeitig wurden dieselben Konzentrationen mit einem Zusatz von 0,005 M ATP, H- und M-Adenylsäure oder Adenosin geprüft. Die normale Teilungsfähigkeit des Materials wurde in einer gleichzeitigen Kontrolle in Seewasser verfolgt.

Eier von *Psammechinus* wurden zunächst in normalem Seewasser befruchtet und dann auf Serien mit DNP oder DNK mit und ohne Zusatz von ATP usw. verteilt.

Tabelle VI a zeigt das Ergebnis eines Einzelversuchs mit ATP und DNP und Tabelle VI b gibt die Mittelwerte aus 3 Versuchen mit *Psammechinus* wieder.

Gleichzeitig war es auch hier deutlich dass in den Lösungen mit ATP die Zellteilungen nicht nur zahlreicher sondern auch regelmässiger und weiter entwickelt waren.

In Versuchen mit *Psammechinus* mit Muskeladenylsäure, Hefeadenylsäure oder Adenosin in Lösungen von DNP oder DNK konnte die hemmende Wirkung von DNP und DNK mit Muskeladenylsäure oder Adenosin teilweise aufgehoben werden. Die Wirkung von Hefeadenylsäure war nicht mit Sicherheit feststellbar. In der Tabelle VII a ist eine Übersicht der Teilungsquoten in den verschiedenen Lösungen auf Grund von Mittelwerten aus 5 Versuchen mit DNP und Tabelle VII b aus 3 Versuchen mit DNK ge geben.

Dinitrophenol und Muskeladenylsäure, Hefeadenylsäure oder Adenosin nach der Befruchtung.

Die Eier wurden zuerst in normalem Seewasser befruchtet und dann in Lösungen von Dinitrophenol oder Dinitrokresol mit und ohne Zusatz von Muskeladenylsäure, Hefeadenylsäure oder Adenosin übertragen.

Tab. VII a.

Psammechinus miliaris.

Zellteilung in % in $6 \cdot 10^{-5}$ M DNP ohne Zusatz	11
Mit Zusatz von 0,005 M Muskeladenylsäure	41
Mit Zusatz von 0,005 M Hefeadenylsäure	10,5
Mit Zusatz von 0,005 M Adenosin	44

Tab. VII b.

Psammechinus miliaris.

Zellteilung in % in $5 \cdot 10^{-5}$ M Dinitrokresol ohne Zusatz	2,0
Mit Zusatz von 0,005 M Muskeladenylsäure	10,6
Mit Zusatz von 0,005 M Hefeadenylsäure	5,1
Mit Zusatz von 0,005 M Adenosin	13,6

Bei Versuchen mit den Eiern von *Echinus* und *Strongylocentrotus* konnte die teilungshemmende Wirkung von DNP oder DNK nur mit Adenosin mehr oder weniger aufgehoben werden. Alle Versuche mit den übrigen Verbindungen führten bei diesen Arten zu keinem positiven Ergebnis.

BESPRECHUNG DER ERGEBNISSE

Die hier mitgeteilten Versuche sind einer einfachen einheitlichen Erklärung noch nicht zugänglich. Die Wirkung ist nicht auf „energiereiche" Phosphatbindungen unmittelbar zurückzuführen, da Adenylsäure oder gar Adenosin sich als ebenso wirksam wie ATP erwiesen haben.

Möglicherweise könnte die Zufuhr der obengenannten Nucleotide oder eines Nucleosids zum Aufbau von ATP in der Zelloberfläche oder im Zellinneren beitragen. Die Ergebnisse der Versuche mit Dinitrophenol und Dinitrokresol erschweren aber eine solche Auffassung. Es erscheint darum notwendig die Untersuchung in verschiedenen Richtungen weiterzuführen.

ZUSAMMENFASSUNG DER ERGEBNISSE

1. Die Wirkung von ATP, Muskeladenylsäure, Hefeadenylsäure und Adenosin auf die Teilung des Seeigeleis wird mit Hilfe der Anwendung von Hemmungsfaktoren studiert.

2. Es zeigt sich dass ATP der teilungshemmenden Wirkung von hypotonischem und hypertonischem Seewasser entgegenwirken kann.

3. Bei Behandlung von Seeigeleiern mit hypo- oder hypertonischen Seewasserlösungen mit und ohne ATP-Zusatz vor der Befruchtung, zeigt sich

nach der Überführung in normales Seewasser und Befruchtung, eine höhere Teilungsquote bei den mit ATP vorbehandelten Eiern.

4. Eier die nach der Befruchtung in normalem Seewasser in hypertonisches Seewasser überführt wurden, zeigten gleichfalls eine grössere Anzahl Teilungen, wenn sie dort nach der Befruchtung mit ATP behandelt wurden.

Bei Anwendung von hypotonischem Seewasser zeigt sich bei der gleichen Methode keine Verbesserung der Teilung durch ATP.

5. In normalem Seewasser befruchtete Eier die danach in hypertonische Lösungen mit Muskeladenylsäure, Hefeadenylsäure oder Adenosin übertragen wurden, zeigten gleichfalls eine höhere Teilungsquote im Vergleich mit den Serien ohne diese Zusätze.

In hypotonischen Lösungen ergaben sich bei Anwendung der gleichen Methode keine Verbesserung der Teilungsquoten mit den obigen Verbindungen.

6. Bei in normalem Seewasser befruchteten Eiern von *Psammechinus* die nach der Befruchtung in Lösungen von Dinitrophenol oder Dinitrokresol mit und ohne Zusatz von ATP, Muskeladenylsäure oder Adenosin überführt wurden bewirkten die letztgenannten Verbindungen eine höhere Teilungsquote.

7. Der Zusatz von ATP, Muskeladenylsäure, Hefeadenylsäure oder Adenosin kann unter den einzelnen beschriebenen Bedingungen nicht nur eine vermehrte Anzahl von Teilungen, sondern auch eine regelmässigere und weitergehende Teilung der Eier bewirken im Vergleich zu den Eiern die durch die direkte Einwirkung der Hemmungsfaktoren mehr oder weniger geschädigt sind.

Am Ende dieser Arbeit ist es mir eine angenehme Pflicht Herrn Prof. John Runnström für die Anregung zu den vorliegenden Untersuchungen und seine Hilfe meinen allerherzlichsten Dank auszusprechen. Weiter danke ich Herrn Prof. Nils Holmgren und Herrn Dr Gunnar Gustafson von der zoologischen Station, Kristineberg, für Ihre freundliche Hilfe und die ständige Beschaffung des für die Untersuchungen benötigten Materials.

LITERATUR

1. Clowes, G. H. A., und Krahl, M. E., *J. Gen. Physiol.*, **20**, 145 (1936).
2. Kaplan, N. O., und Lipmann, F., *Federation Proc.*, **6**, 266 (1947).
3. Kriszat, G., und Runnström, J., *Trans. N. Y. Acad. Sci. Ser. II*, **5**, 162 (1951).
4. Lipmann, F., *Federation Proc.*, **8**, 597 (1949).
5. Loomis, W. F., und Lipmann, F., *J. Biol. Chem.*, **173**, 807 (1948).
6. —— *ibid*, **179**, 503 (1949).
7. Runnström, J., *Advances in Enzymol.*, **9**, 241 (1949).
8. Runnström, J., und Kriszat, G., *Expll. Cell Res.*, **1**, 284 (1950).
9. —— *ibid.*, **1**, 497 (1950).
10. Wicklund, E., *Arkiv Zool.*, **42 A**, 11 (1949).

THE ACTIVATION OF THE EGG OF THE SEA URCHIN *PSAMMECHINUS MILIARIS* BY PERIODATE

GEORG KRISZAT and JOHN RUNNSTRÖM

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

Received December 5, 1951

Jelly coat substance, heparin, blood group H-substance and fucoidin have been shown to inhibit the fertilization or at least the membrane formation in the sea urchin egg, see also (2, 3, 5, 17, 22). The inhibitory substances are polysaccharides or contain polysaccharides as their main component. The inhibitory action of these substances was easily removed by addition of sodium periodate (2, 17).

Starting from these results, the present writers (17) applied periodate directly on the egg surface. In underripe eggs the membrane formation is rather poor and polyspermy is often noted. Upon pretreatment of these eggs with 5.10^{-5} N sodium periodate in sea water, the fertilization occurred in a normal way and the further development was much improved.

Moreover, the demonstration was given that periodate pretreatment sensitizes the unfertilized mature eggs to the activating effect of a hypertonic solution. An exposure which gave no effect in non pretreated eggs caused a high percentage of activation in eggs previously subjected to the action of periodate. A certain percentage (10–15) of the eggs proved to be activated merely as an effect of periodate pretreatment. In the present work an attempt was made to increase the extent of activation following upon periodate treatment. The mere removal of the jelly coat surrounding the egg proved to render the eggs much more sensitive to the activating effect of periodate. The experiments with periodate were carried out in presence or absence of Ca with the aim at elucidating the effect of its action.

MATERIAL AND METHODS

The material used was the littoral form of *Psammechinus miliaris*. The eggs were generally kept in sea water with a salinity of 25 per mille. In most of the experiments the jelly coat was removed prior to the treatment with periodate. For this purpose the eggs were washed for 10 minutes in sea water, the pH of which was lowered to 4.5 by addition of HCl. Sodium periodate and potassium iodate were used in concentrations of 6.10^{-5}–9.10^{-5} N. For each series of experiments fresh sea water solutions

were prepared from a 0.1 N Na-periodate distilled water stock solution by dilution with sea water (2, 17). Ca-free sea water was prepared according to the table published by Öhman (13) and then diluted with redistilled water to 25 per mille.

For the experiments with Ca-free sea water eggs without jelly coat were used. Before being transferred to Ca-free sea water the eggs were first washed twice with isotonic sodium citrate solution and afterwards with Ca-free sea water to remove small amounts of Ca.

Two criteria were employed in order to ascertain the number of activated eggs by treatment with periodate or iodate. In the first method the number of eggs with elevated membranes was counted immediately after the treatment. In the second method the eggs were transferred after some time to hypertonic medium (0.6 ml 2.5 N NaCl + 2 ml sea water). Thereafter the number of eggs showing angular plasmolysis was evaluated. This type of plasmolysis is characteristic of activated eggs, for references see (16). Samples of 200–300 eggs were examined. The figures in the table represent the highest and the lowest average values of per cent angular plasmolysis in five samples. Furthermore the average value for the whole material is given.

RESULTS

Eggs with their jelly coat were removed from the ovaries and were immediately subjected to 6.10^{-5} N sodium periodate in sea water; 10–25 per cent of these eggs showed an activation, see Table I b. After removal of the jelly coat a similar treatment gave an activation of 50–60 per cent of the eggs, see Table I a.

Elevation of the fertilization membrane occurred 2–3 minutes after exposure to periodate. As shown in Fig. 1 the eggs in which membrane formation occurs, present a slightly contracted state of the surface. The eggs without membrane formation on the other hand were more perfectly spherical than the control eggs. Fig. 2 represents normal unfertilized eggs after about 3 minutes' exposure to the hypertonic medium. The fine wrinkling of the surface is well visible. Furthermore the eggs are mostly not spherical, but somewhat elongated in one direction. Fig. 3, on the other hand represents eggs after exposure for about 3 minutes to hypertonic medium subsequent to treatment with 6.10^{-5} N Na-periodate; see Table I a. A number of eggs present here the angular plasmolysis and were thus classified as activated eggs. It is interesting, however, that also the other eggs differed in an obvious way from the non pretreated eggs of Fig. 2. They were quite smooth and in general perfectly spherical. In the non pretreated eggs this state was attained only after 30–40 minutes' exposure to the hypertonic medium. The pretreatment with periodate changed the eggs so that the state of smooth surface and spherical form was obtained immediately upon transfer to the hypertonic medium.

Table I

The effect of Na periodate and iodate on the unfertilized egg of *Psammechinus miliaris*. Eggs deprived of the jelly coat in a and c–f.

Solution	a	b	c	d	e	f
	$6 \cdot 10^{-5}$ N Na-periodate in sea water	$6 \cdot 10^{-5}$ N Na-periodate in sea water	$9 \cdot 10^{-5}$ N Na-periodate in sea water	$6 \cdot 10^{-5}$ N Na-periodate in Ca-free sea water	$6 \cdot 10^{-5}$ N K-iodate in sea water	$9 \cdot 10^{-5}$ N K-iodate in sea water
Activated eggs in %	50–60	10–25	60–90	30–50	1–5	10–20
Average in %	57	17	78	41	2	11

Activated eggs are more strongly stained with methylene blue than are non activated (15). A striking difference was also observed when non pretreated and periodate pretreated eggs were compared. As is obvious from Fig. 4 and 5, the pretreated eggs take the stain more strongly than the non pretreated eggs. However, the pretreated eggs with no membrane take the stain much less intensively than those with a membrane formed. The former have a more perfectly spherical shape than the non pretreated eggs. They correspond no doubt to the smooth spherical eggs observed upon transfer to hypertonic medium. In these eggs certain cortical changes have thus occurred, but they do not bring the eggs to the fully activated stage in which increase of methylene blue staining also occurs.

A monaster developed in the activated eggs. A number of these also underwent cleavage until 2 and 8-cell stages. An increased number of activated eggs cleaved upon transfer to sea water with 0.005 M ATP. These experiments will be repeated and extended before being reported in detail.

As follows from the Table I a and c the number of activated eggs depended also on the periodate concentration. Treatment with potassium iodate in the same concentrations as in the periodate experiments gave only a low percentage of activation, see Table I e and f.

It was also possible to activate eggs by periodate in Ca-free sea water, see Table I d. In these experiments a fertilization membrane was elevated, but it was only very weakly refractile and disappeared after a few minutes. Furthermore, the eggs did not present the contraction of the surface visible in the activated eggs of Fig. 1. If, however, the eggs were transferred to normal sea water immediately upon membrane elevation, they often exhibited surface contraction of short duration. Thereafter the membrane became more refractile and more resistent.

DISCUSSION

As demonstrated above, an activation of *Psammechinus miliaris* eggs occurs under the action of periodate in rather low concentrations. Iodate has only a weak action in the same concentration range.

It seems rather probable that periodate at the concentrations, temperature and pH prevailing in these experiment acts on polysaccharides in the surface of the egg. Morgan and Watkins (11) studied the action of periodate on the surface of human erythrocytes. They found an inactivation of a number of different blood group receptors. They state as probable that mainly receptors of carbohydrate nature are attacked by the periodate.

The periodate treatment of the unfertilized mature eggs thus probably breaks down a carbohydrate component present in the egg surface. This breakdown may be the first link in the activation process. The demonstration of the inhibitory action exerted by certain polysaccharides on fertilization or membrane formation (2, 3, 17) lends further support to this conception. It is particularly significant that rather weak concentrations of periodate removed the action of the inhibitors.

The rather varied carbohydrase activity found by Hultin and Lundblad (6) in the sea urchin spermatozoa is a further piece of evidence for the role of carbohydrates in the activation, see also (8, 24). Finally Monné and Slautterback (9) demonstrated the presence of carbohydrates in the cortical layer by means of histochemical methods.

By fractional centrifugation, Moskowitz and Calvin (12) isolated a component of the erythrocyte surface called elinin. It is a lipid-carbohydrate-protein complex which contains the RH, A and B blood group factors. Elinin appears as a rodlike molecule, with a molecular weight of about 4.10^7. Possibly, molecular complexes of a similar kind may be constituents of the sea urchin egg surface.

It has been shown above that the activation is lower in eggs with the jelly coat on than in those devoid of their coat. As known from Vasseur's chemical work (23) the jelly substance reacts readily with periodate, see also (17). The results suggest that there is a substance similar, but probably not identical with the jelly substance present in the egg surface.

It is known that water extracts from spermatozoa cause an agglutination of the naked eggs as well as of the eggs provided with jelly coat (1). Clupein acts in the same way as the sperm extract (19) which seems to exert its action in virtue of its content of basic protein (7). Clupein and sperm ex-

tract proved to have an activating effect on the egg (21, 25). In the clupein treated eggs a low granular membrane appeared, the nucleus migrated toward the centre of the egg and a monaster became visible. A certain cleavage was also observed. Clupein treated eggs exhibited the angular type of plasmolysis when immersed in a hypertonic solution. Wicklund (25) demonstrated the great difference between the organization of the unfertilized control eggs and that of the clupein treated eggs.

The similarity between the results of the clupein and that of the periodate treatment is obvious. There are also certain differences. As already mentioned the fertilization membranes formed upon clupein treatment are always granular and poorly elevated. Subsequent to periodate treatment, on the other hand, well developed membranes may appear.

The agreement in the action of basic proteins and of periodate may reside in their common action on acid polysaccharides in the egg surface. The former precipitate, the latter depolymerize the polysaccharides. In both cases an inhibitory effect of the polysaccharides may be removed and cause the observed activation. Even if the periodate fails to activate the eggs, it causes a certain change in the egg surface. This is demonstrated by the smooth plasmolysis and spherical shape found in the non activated periodate treated eggs. This contrasts with the wrinkled surface and the asymmetrical shape of the control eggs. Runnström and Monné (20) studied the effect of different temperatures on *Psammechinus* eggs. When kept at 26.2° for 135 minutes the eggs shrank with a smooth surface upon immersion in hypertonic medium. On prolonged exposure to 26.2° an increasing number of eggs exhibited angular plasmolysis, when submitted to the hypertonicity test. This was an indication of accomplished activation. In a parallel sample kept at 17° for the same time the eggs presented upon hypertonic treatment a wrinkled surface and an asymmetrical shape as shown in Fig. 2. Smooth plasmolysis in hypertonic solution reflects probably a first step in the activation process.

The present writers entertain the idea that the wrinkled surface of unfertilized eggs in hypertonic medium as shown in Fig. 2 depends on the existence of polymerized complexes of "elininlike" molecules which particularly adhere to each other by their carbohydrate components. By the change occurring upon exposure to periodate the "cement" substance breaks up and the rodshaped molecules can be more easily deplaced in relation to each other, when the eggs shrink in the hypertonic medium. Consequently the surface becomes smooth. The angular plasmolysis represents a stage in which a rearrangement has taken place. New attachment points now bring

about a three dimensional framework. In this the rodlike molecules are more finely dispersed than in the aggregates present in the non activated egg (16, 18). A transition from a coarser linear to a more dispersed three dimensional arrangement of rodlike molecules would also explain the decrease of birefringence of the cortex (10, 14) and that of light scattering of the interior cytoplasm (16) which attend the activation of the egg. The outlined picture accounts rather well for the decreased readiness of inner crystallization observed to prevail after fertilization (18).

It is of interest that the effect of periodate and of sperm attachment are in a way additive. As mentioned in the introduction, subthreshhold treatment with periodate may improve the effect of fertilization (17). This tends to indicate that periodate treatment primarily changes the same component as that involved in the initial step of normal activation.

It is known (4) that Ca plays an important role in the activation of the egg. It was therefore of interest to find that periodate was able to activate the eggs even in absence of Ca. The periodate action probably replaces a natural process in which Ca is necessary. The contraction of the egg surface and the toughening of the membrane failed, however, to occur in periodate treated eggs in absence of Ca. This ion is also necessary for developing the properties of the cortex manifested in the angular plasmolysis, for references see (16).

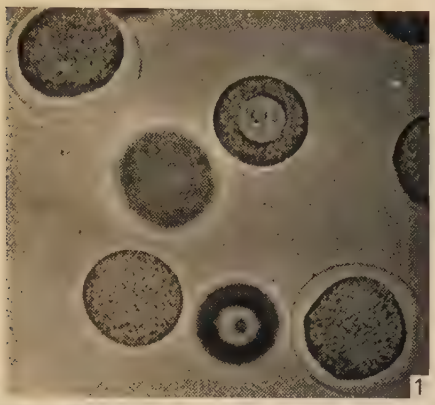
SUMMARY

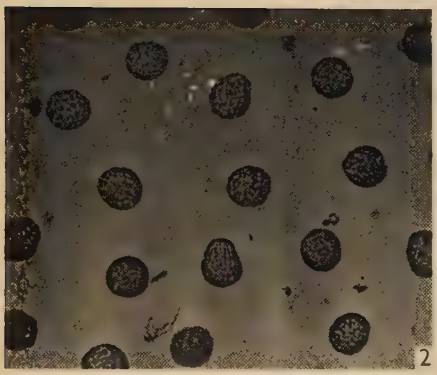
Sodium periodate in concentration 6.10^{-5}–9.10^{-5} N has an activating effect on the eggs of *Psammechinus miliaris*. A membrane elevation occurred and cleavage started. It did not proceed beyond the 2–8 cell stage.

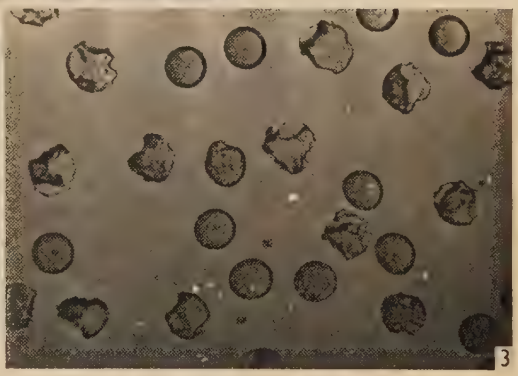
The eggs activated subsequent to the periodate treatment exhibited the angular plasmolysis characteristic of fertilized eggs when immersed in a hypertonic medium.

Even non activated periodate treated eggs behave differently from non pretreated eggs. The latter are often somewhat elongated in one direction and show a fine wrinkling on the surface. The former are spherical with a smooth surface. This behaviour indicates that the eggs have taken a step towards activation. Eggs activated subsequent to periodate treatment are as strongly stained with methylene blue as are fertilized eggs. In contrast the non activated pretreated or the non pretreated unfertilized eggs are very weakly or not at all stained with methylene blue.

Iodate of corresponding concentrations has a much lower activating effect than sodium periodate.







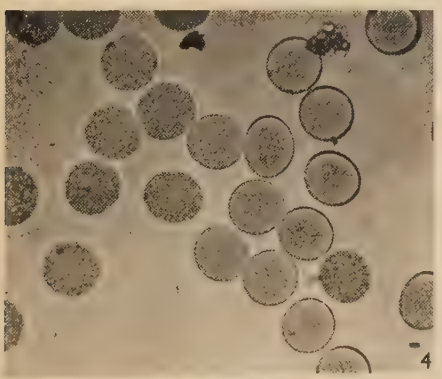


Fig. 1. Eggs activated upon exposure to Na periodate.
Fig. 2. Effect of hypertonic sea water on non pretreated control eggs.
Fig. 3. Effect of hypertonic sea water on eggs pretreated with Na periodate.
Fig. 4. Non pretreated control exposed to methylene blue 1:10 000 in sea water.
Fig. 5. Eggs pretreated with Na periodate exposed to methylene blue 1:10 000 in sea water.

The activation of the eggs by periodate occurred even in absence of Ca. After transfer to Ca-containing medium the eggs underwent a contraction and the membrane became much more refractile.

This work was supported by the Swedish Natural Science Research Council. We are greatly indebted to the authorities of The Kristineberg Zoological Station for help and supply of material.

REFERENCES

1. Frank, J. A., *Biol. Bull.*, **79**, 371 (1940).
2. Harding, C. V., *Exptl. Cell Res.*, **2**, 403 (1951).
3. —— *Arkiv Zool.* (1952) (In press.)
4. Heilbrunn, L. V., Colloid Chemistry of Protoplasm. Protoplasma Monographien 1, Berlin, 1928.
5. Heilbrunn, L. V., and Wilson, W. L., *Proc. Soc. Exptl. Biol. Med.*, **70**, 179 (1949).
6. Hultin, E., and Lundblad, G., *Exptl. Cell Res.*, 3, 427 (1952).
7. Hultin, T., *Pubbl. Staz. Zool. Napoli*, **21**, 153 (1947).
8. Lundblad, G., and Monroy, A., *Arkiv Kemi*, **2**, 343 (1950).
9. Monné, L., and Slautterback, D. B., *Exptl. Cell Res.*, **1**, 477 (1950).
10. Monroy, A., and Montalenti, G., *Biol. Bull.*, **92**, 151 (1947).
11. Morgan, W. T. J., and Watkins, W. M., *Brit. J. Exptl. Path.*, **32**, 34 (1951).
12. Moskowitz, M., and Calvin, M., *Exptl. Cell Res.*, **3**, 33 (1952).
13. Öhmann, L. O., *Arkiv Zool.*, **36 A**, No. 7 (1944).
14 Rothschild, Lord, and Swann, M. M., *J. Exptl. Biol.*, **26**, 177 (1949).
15. Runnström, J., *Arkiv Zool.*, **7**, No. 13 (1911).
16. —— *Advances in Enzymol.*, **9**, 241 (1949).
17. Runnström, J., and Kriszat, G., *Exptl. Cell Res.*, **1**, 355 (1950).
18. —— *Arkiv Zool.* (1952) (In press).
19. Runnström, J., Lindvall, S., and Tiselius, A., *Nature*, **153**, 285 (1944).
20. Runnström, J., and Monné, L., *Arkiv Zool.*, **36**, No. 20 (1945).
21. Runnström, J., Monné, L., and Wicklund, E., *J. Colloid. Sci.*, **1**, 421 (1946).
22. Runnström, J., and Wicklund, E., *Arkiv Zool.*, **1**, No. 13 (1950).
23. Vasseur, E., *Acta Chem. Scand.*, **4**, 1144 (1950).
24. —— *Exptl. Cell Res.*, **2**, 144 (1951).
25. Wicklund, E., *Arkiv Zool.*, **40**, No. 5 (1947).

BRIEF NOTES

NOTE ON THE ORIGIN OF THE CORTICAL GRANULES IN *ARBACIA PUNCTULATA* EGGS[1]

DAVID McCULLOCH[2]

Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass.

Received December 3, 1951

In 1945, Runnström and Monné (3), in a study of maturation changes in the eggs of certain European sea urchins, observed that the cortical granules, which were identified by their staining reactions with iron hematoxylin after Flemming fixation, are identifiable in the cortex of *Psammechinus miliaris* and *Brissopsis lyerifera* eggs at all times after the completion of maturation. Further observation of *Brissopsis* oocytes, however, indicated that bodies having the staining properties of cortical granules could be found in the cytoplasm of the egg in a stage slightly earlier than that of germinal vesicle breakdown. It was suggested that the presumptive cortical granules may be formed in the cytoplasm and arrive at their cortical site by "migration." Recently (1951), Monné and Hårde (1) have continued this study, and have confirmed the earlier result, not only in *Brissopsis*, but also in *Psammechinus*, *Echinocardium cordatum*, *Paracentrotus lividus*, and *Arbacia lixula*. They state that "the prospective cortical granules of the oocytes begin to move in a centrifugal direction a short time before the breakdown of the germinal vesicle" and that the movement is complete by the time the polar bodies are expelled. They postulate that the mechanism of this movement is an expanding condensation of the ground cytoplasm beginning in the vicinity of the germinal vesicle remnant and spreading peripherally with time, the cortical granules being squeezed out into successive positions nearer and nearer the surface. The cortical granules are thought to contain mucopolysaccharide in significant amounts.

During the course of an electron microscopic study of the egg of the American sea urchin, *Arbacia punctulata*, evidence on the origin and structure of the cortical granules in this species has been obtained, as will be described below.

Moser (2) has previously demonstrated that mature *Arbacia punctulata* eggs, like those of other sea urchins, contain cortical granules, and that these are released on fertilization. Electron micrographs of thin sections of fixed, unfertilized, mature *Arbacia* eggs show some details of the structure of these bodies (Fig. 1). They seem to consist of a number of spherical units (c) 0.2–0.3 microns in diameter, "embedded"

[1] This work was done during tenure of an Atomic Energy Commission Predoctoral Fellowship in Biology, and is an extract from the author's Doctoral Thesis.

[2] Present address: Department of Zoology, University of Chicago, Chicago 37, Ill.

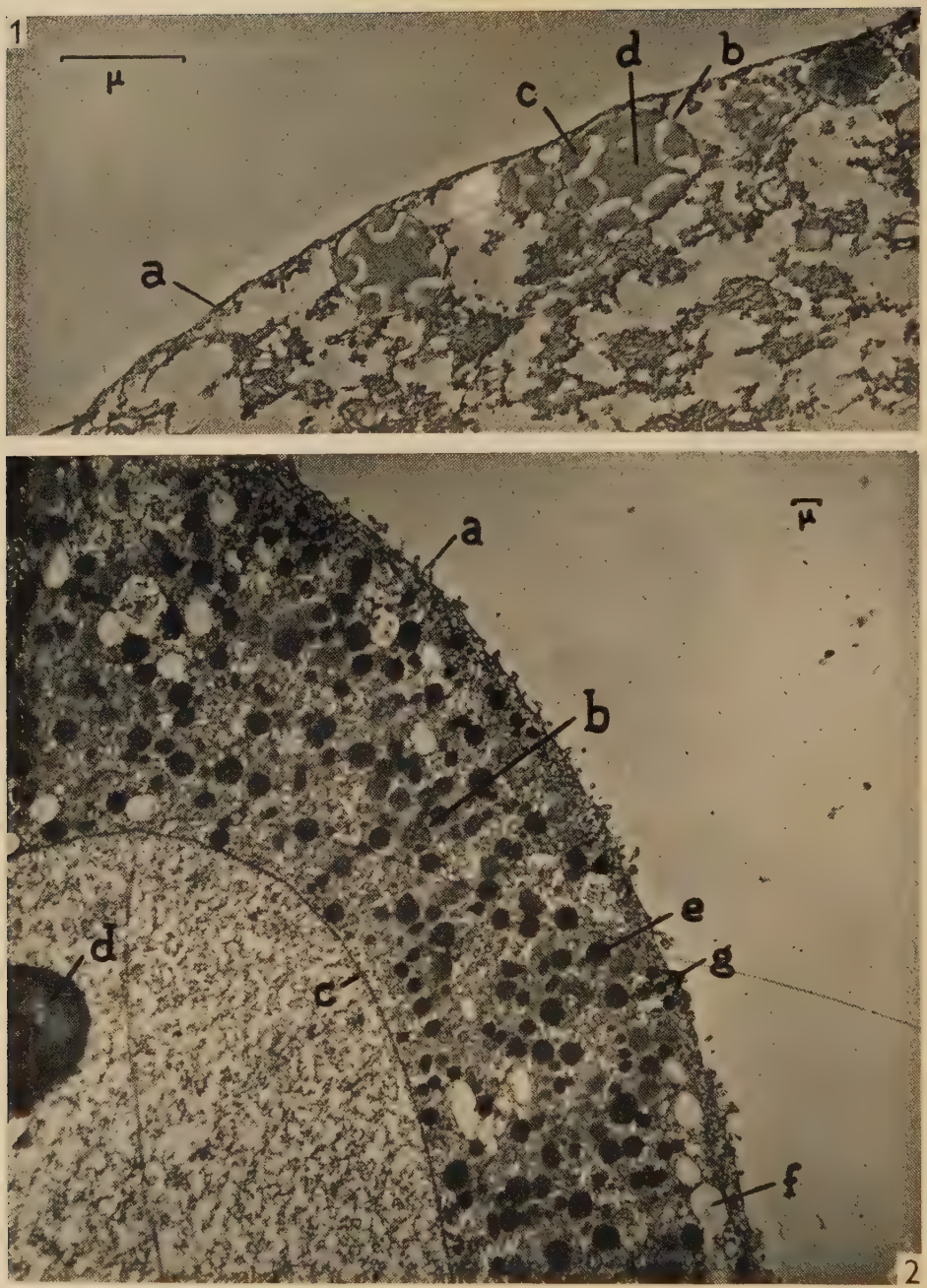


Fig. 1. Electron micrograph of thin section through cortex of mature, unfertilized egg. a) cell surface; b) cortical granule membrane; c) 0.2–0.3 micron bodies; d) matrix. 16 500 ×.

Fig. 2. Electron micrograph of thin section of *Arbacia* oocyte. a) surface projections; b) cortical granule; c) germinal vesicle surface; d) nucleolus; e) yolk; f) pigment spaces (pigment has dissolved on fixation); g) medium sized granules, including mitochondria and oil. 3700 ×.

in a matrix substance (d) having much the same appearance as the substance of the spherical units, the whole being surrounded by a very thin "membrane" (b) of the order of 200–300 A in thickness which contacts the immediate cell surface. This morphology is best preserved after fixation in a 0.3 per cent osmium tetroxide —0.4 per cent chromic acid mixture in sea water (6 hours), as shown in Figure 1. After 0.3 per cent osmium tetroxide fixation, or after formalin fixation, the small spherical units seem not to have been preserved, and the central matrix remains as an often badly shrunken mass in the center of the cortical granule. In all fixatives the surrounding membranous structure is present. Whether it represents a membrane belonging to the cortical granule or a special sort of cytoplasmic condensation in this location on fixation of course cannot be decided from the evidence. The substructure of the fixed cortical granule described above may not exactly represent the true sub-structure, but the morphology described is diagnostic for cortical granules, none of the other particulates of the egg having a similar appearance. In the mature, unfertilized egg, the cortical granules have been found only in intimate contact with the cell surface and nowhere else in the cell.

Examination of electron micrographs of immature *Arbacia* eggs in sections in which the germinal vesicle could be seen, as well as in others, showed that structures having the morphology of cortical granules could be found within the cytoplasm (Fig. 2 (b)). None, however, were present in the "cortical layer," the outer micron or so of the cell. It thus appears that the cortical granules exist in the interior cytoplasm prior to the breakdown of the germinal vesicle, and that there must occur during maturation the same sort of reaction as that described by Monné and Hårde, a reaction which selectively throws the presumptive cortical granules into the cortex of the egg. A detailed study of this reaction is feasible by electron microscopy and should prove highly interesting.

The surface of the oocyte has been found not to be smooth like that of the mature egg, but to contain numerous fine projections (a) which appear to be protoplasmic protrusions from the surface of the cell (Fig. 2). This observation agrees with the findings of Runnström and Monné (3), and others, in sea urchin oocytes of other species. In Fig. 2 can also be seen the surface of the germinal vesicle (c) and the nucleolus (d). It is thus evident that in the *Arbacia* oocyte, cortical granules are found in the cytoplasm even before the breakdown of the nucleolus, and hence that the formation of these bodies does not occur as a consequence of nuclear breakdown, but as a result of some earlier process in oogenesis.

REFERENCES

1. Monné, L., and Hårde, S., *Arkiv Zool.*, *Ser.* **2**, **1**, 31 (1951).
2. Moser, F., *J. Exptl. Zool.*, **80**, 423 (1939).
3. Runnström, J., and Monné, L., *Arkiv Zool.*, **36 A**, No. 18 (1945).

IMPROVEMENT OF AUTORADIOGRAPHIC RESOLUTION BY INCINERATION OF HISTOLOGICAL SECTIONS

ERIK ODEBLAD

Hormone Research Laboratory, Women's Clinic, Karolinska Institutet, Sabbatsberg Hospital, Stockholm, Sweden

Received January 25, 1952

According to Doniach and Pelc (2) and Gross *et al.* (4), the autoradiographic resolution improves, following a reduction of the thickness of the histological section. Attempts have here been made to bring about these conditions by incineration of sections. In microincineration it is advisable to avoid the dislocation of the constituents of the ash (3). The following requirements must be fulfilled: 1) The histological structure must be maintained to a sufficient degree. This was controlled by examining the microscopical picture in the course of the incineration. 2) Radioactivity must not be loosened by votalization which was controlled before and after the incineration (P^{32}) by aid of a Geiger-Müller tube. 3) A considerable reduction of the section thickness must occur. This was controlled by micrometer measurements on cross surfaces of sections (broken specimens). 4) The technique must include low-atomic support slides to avoid scattering of beta particles.

Deparaffinized 5μ sections were used, mounted on 10μ mica foils. During exposure the foil was supported by a methacrylate slide (average atomic number 3.2), and was pressed against the emulsion (no-screen X-ray film or stripping film).

For incineration an automatically controlled furnace (Grave Co) was used, and +550° C was found to be a suitable temperature. The mica foil with the section was placed in the warm furnace, and the procedure of incineration was finished within 4 minutes. Later on, after some training, satisfactory results could sometimes be obtained by heating the foil over a gas jet, until the stage of carbonization was passed.

By comparing autoradiographs of the same section before and after incineration, a considerable gain in resolution was often found. However, the micro-geometrical relationships during the autoradiographic exposure appear difficult to control after incineration, leading to inconstant resolution and non-quantitative results. Nevertheless, certain new qualitative informations (1) may be obtained with autoradiography after incineration.

REFERENCES

1. Aminoff, U., Magnusson, G., Odeblad, E., and Wetterdal, K.-H., *Exptl. Cell Res.* **3**, 609 (1952).
2. Doniach, I., and Pelc, S. R., *Brit. J. Radiol.*, **23**, 184 (1951).
3. Glick, D., Techniques of histo- and cytochemistry. Interscience, New York, p. 140–147, 1949.
4. Gross, J., Bogoroch, R., Nadler, N. J., and Leblond, C. P., *Am. J. Roentgenol. Radium Therapy*, **65**, 420 (1951).

HIGH JUXTAMEMBRANOUS INCORPORATION OF P^{32} OF THE UNFERTILIZED RABBIT OVUM

ULLA AMINOFF, G. MAGNUSSON, E. ODEBLAD and K.-H. WETTERDAL

Hormone Research Laboratory, Women's Clinic, Karolinska Institutet, Sabbatsberg Hospital, Stockholm, Sweden

Received January 25, 1951

In a previous report (2) it was demonstrated that the granulosa tissue of Graafian follicles incorporates considerably more P^{32} than the ovum after injection in rabbits of labeled phosphate. This has been confirmed by further experiments also to be the case in growth follicles 2–48 hours after injection. A high juxtamembranous incorporation of P^{32} in the ovum was also found. To study this last phenomenon more thoroughly, the resolution of our technique (20 μ) was increased (to about 8 μ) by means of incineration (3).

Experimental. Two anoestrous female rabbits were given 1 mC P^{32}/kg of body weight intravenously as orthophosphate and were killed 24 hours later. Four adult mice were given 30 μC P^{32}/g of body weight and were killed after 48 or 96 hours. The ovaries were then fixed in 6 per cent formalin containing 0.5 per cent trichloracetic acid for 24 hours at +19° C. Paraffin embedding and sectioning in 10 μ (rabbits) and 5 μ (mice) sections were undertaken, thereby applying an incineration procedure (3). After exposure, the Gevaert Dentus Rapid film was developed for 30 minutes in Kodak DK 20 with KBr added to 0.2 per cent (density and grain number proportional to exposure). The silver of the reverse side was removed with Farmers solution. Grain countings were performed in oil immersion.

Table I

Grains per 14 μ^2 corresponding to various structures within the growth follicle.

Follicle nr	Ovum (I)	Juxtamembranous ring (II)	Granulosa tissue (III)	Surrounding tissue (IV)	(II) minus (III) (V)	P (V)
1	0.64±0.11	2.64±0.10	1.41±0.15	1.27±0.14	1.23±0.18	< 0.001
2	0.37±0.12	3.57±0.30	1.92±0.20	0.96±0.15	1.65±0.36	< 0.001
3	0.57±0.30	2.24±0.20	1.88±0.18	0.91±0.13	0.36±0.27	0.2
4	0.83±0.13	3.15±0.25	1.96±0.18	1.06±0.13	1.19±0.27	< 0.001
5	1.04±0.13	4.30±0.31	2.73±0.22	1.14±0.39	1.57±0.40	< 0.001
6	0.75±0.12	4.00±0.30	2.42±0.21	1.65±0.16	1.58±0.37	< 0.001
7	0.83±0.32	5.48±0.50	2.13±0.36	1.46±0.33	2.35±0.62	< 0.001

Results. In 13 out of 55 vital growth follicles with an ovum cut in the centre, a high juxtamembranous incorporation was visible on the autoradiographs. In 27 follicles the incorporation was doubtful, in 15 no incorporation could be seen. Table I

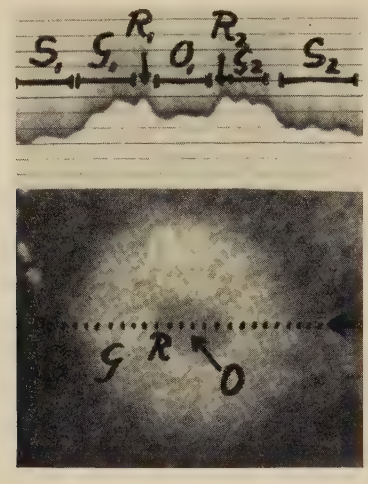


Fig. 1. Autoradiograph of growth follicle of rabbit, enlarged 46 times, and a corresponding photometer curve. Dotted line = path in photometer recording. O = ovum, R = ringformed high juxtamembranous incorporation of P^{32}, G = granulosa tissue and S = surrounding tissue. O_1; R_1, R_2; G_1, G_2; and S_1, S_2 indicate the corresponding parts on the photometer curve.

shows the results of grain countings. There is a statistically significant difference between the juxtamembranous grain numbers and those of the granulosa tissue. An enlarged autoradiograph and a corresponding photometer curve (fig. 1) illustrate the high juxtamembranous incorporation.

Since it was difficult to control the exact geometrical relationships during the exposure, no conclusions may be drawn about the actual concentration of P^{32} in the various parts of the sections.

Discussion. The observed high juxtamembranous incorporation of P^{32} may be due to geometrical artefacts (peaks of the section in close contact with the emulsion). This, however, could be excluded in microscopic examination by shadowing in light with glancing incidence and on cross surfaces of incinerated sections. The high juxtamembranous incorporation may thus be regarded as real, but was not definitely localized, owing to the limited resolution (8μ). It may reside in the *zona pellucida*, immediately beneath the surface of the ovum or in the inner layer of granulosa cells.

The dissolving effect by the histological treatment is discussed elsewhere (4).

It is of interest to compare our findings with Lindberg's results (1), concerning a surface-bound metabolism of phosphates in unfertilized sea urchin eggs.

REFERENCES

1. Lindberg, O., *Exptl. Cell Res.*, **1**, 105 (1950).
2. Odeblad, E., *ibid.*, **2**, 574 (1951).
3. —— *ibid.*, **3**, 608 (1952).
4. —— *Acta Radiol.*, Suppl. 92 (1952).

A TECHNIQUE FOR SECTIONING TISSUE CULTURES

ERIK ENGHUSEN

Biological Institute, Carlsberg Foundation, Copenhagen, Denmark

Received January 25, 1952

As to the sectioning of tissue cultures Parker (1) describes the following technique: "The paraffin block is trimmed in such a manner that flat sections of the entire culture may be obtained." Anyone trying this may meet with difficulties. The technique described below has been employed for the past five years and has been found to be a most satisfactory solution to the problem.

As to the fixing of the culture, it has proved convenient to let the tissue remain in its culture medium on the slide or in the flask to avoid deformation caused by contraction. Slide cultures are fixed in Ringer-formol for one hour. After being washed under the tap, the culture is slightly stained so that it may be more easily seen after being embedded in paraffin. A very dilute hematoxylin is used. The staining is interrupted when a slight colouring is obtained, and the culture is then rinsed in water to remove excess of stain. It is then dehydrated for three to five minutes in 70 per cent alcohol and for five minutes in pure alcohol. Excess alcohol is removed with filter paper. The culture is then cleared in benzene until it becomes transparent. Benzene is chosen as a medium since it readily allows the paraffin to penetrate the tissue. The culture is now removed from the slide in the usual way with a cataract knife, placed in melted paraffin and left in the paraffin-oven for one to two hours. Paraffin with a melting point between 45–50° is used. The culture is thereupon transferred to fresh paraffin and left there for an hour. It may be kept there for as much as 24 hours without harm.

A paraffin block 2 cm square is mounted and placed in the microtome, where it remains during the whole of the following process. First, the block is trimmed with the microtome knife so as to obtain a level surface which will serve to support the culture. It is recommended for this type of work to use a microtome knife whose object-holder is provided with a pinion, since it is necessary during the process to be able to raise and lower the block without at the same time disturbing its position in relation to the knife. After the object-holder has been lowered, the culture is transferred from the melted paraffin to the level surface of the paraffin block with the aid of a slightly warmed scalpel. One or two drops of warm paraffin are then poured onto the culture to cover it with a layer of paraffin less than one mm thick. As the culture is still not parallel with the plane-surface a hot scalpel is held over the embedded culture, for a short time, to melt the uneven paraffin-layer which covers it. Great care should be observed in doing this to avoid melting the smooth paraffin-layer under the culture. After the paraffin has solidified slightly, without disturbing the block, the microtome knife is moved out just enough to ensure that the culture lies behind and under the cutting-edge. The block is then raised sufficiently so that the surface of the paraffin which covers the culture lies about 0.2 mm higher than

the knife-edge. The knife is then moved slowly backwards over the culture. In this way the knife smoothes the soft paraffin and simultaneously presses the culture down gently against the underlying plane. There is no danger of damaging or loosening the culture, if, at the same time, the block's pressure on the knife is regulated with the aid of the pinion. The smoothing process can be repeated until a mirror-like surface is obtained. At this stage it is easy to see the culture, which has already been stained with hematoxylin.

The culture is now ready to be cut into sections in the usual way. 4–6 serial sections can be obtained from slide cultures. When flask-cultures are used the best results are gained by adding the reagents to the culture *in situ*. After dehydration it is simple enough to separate the culture from its medium, but difficult to remove the hardened culture through the narrow neck without destroying the tissue. For this reason it is preferable to use Carrel flasks with removable bottom, so avoiding the necessity of cutting the tops off with a glass-cutter. The slight coloration does not adversely affect the subsequent staining with hematoxylin-eosin.

REFERENCE

1. Parker, R. C., Methods of tissue culture. New York, p. 250, 1950.

INCORPORATION OF C^{14}-LABELED CARBON DIOXIDE INTO THE PROTEINS OF DEVELOPING SEA URCHIN EGGS

TORE HULTIN and GUNILLA WESSEL

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

Received March 13, 1952

Egg material: Eggs from the sea urchin *Psammechinus miliaris* (Gmelin) were used in the experiments.[1] Dilute suspensions of eggs were fertilized and allowed to develop at 20° C in glass trays (11). At least 90 per cent of the ripe eggs developed into normal plutei. Jelly-free eggs were used in all experiments.

Isotope incubations: Equal samples were taken from the egg suspensions 1) just before fertilization, and 2) at 3 $^1/_2$ hour intervals following fertilization. The samples were washed twice in CO_2-free sea water and then transferred to a known volume of the same medium, which was buffered by 0.01 M glycyl-glycine (18) to a pH of 7.1 or 8.1. The isotope was supplied in the form of $NaHC^{14}O_3$ (0.122 mM/mC). The egg samples within a single series of experiments were incubated with equal amounts of isotope. The isotope concentration range was 2–5 μCs in 5 ml of sea water per 1 mg of total egg nitrogen. The incubations were performed in closed glass tubes with appropriate agitation. All samples were incubated with isotope for 3 $^1/_2$ hours. After the incubations, the eggs were washed in isotonic NaCl-solution, frozen and stored at —20° C. In all, seven such series of experiments were performed.

Preparation of carbon samples: The frozen material was extracted with cold 10 per cent trichloroacetic acid (TCA), lipid solvents, and 5 per cent TCA at 90° C (17). In two series, the frozen eggs were extracted 3 times with 0.3 M KCl. These dilute extracts were centrifuged at high speed until almost completely free from opalescence. The extracts and the sediments were then treated as described above. All extractions were made by grinding the material with an air-driven, rotating glass-rod.

In order to avoid interference from any polysaccharide impurities (14) remaining in the protein residues, these fractions were hydrolyzed with 6.5 N HCl at 120° C for 10 hours. The amino acids were decarboxylated by ninhydrin treatment (21) and the specific activities of the carboxyl carbon was measured in the form of $BaCO_3$.

The extracts with cold TCA were often slightly opalescent (7). This opalescence was probably due to material released from the yolk granules (8), which are partially decomposed by the freezing procedure. If such extracts were carefully treated with ether, the opalescence could be removed as a precipitate. The clear solutions were then thoroughly extracted with ether in a liquid-liquid extractor in order to remove the TCA. Finally, the solutions were acidified with HCl and evaporated on a steam bath. The solid residues were combusted, and the specific activities were determined.

[1] The experiments were performed at the Zoological Station of Kristineberg, Sweden, during 1950–1951.

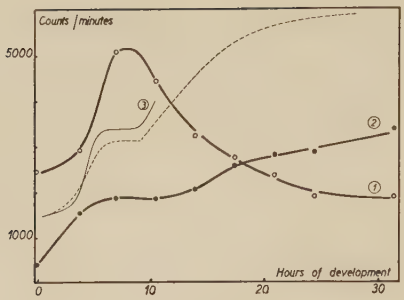


Fig. 1.

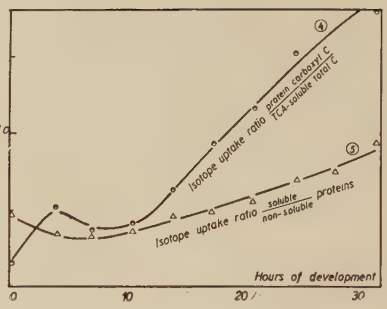


Fig. 2.

Fig. 1. 1) Rates of isotope uptake into the TCA-soluble fraction (total C) at different stages of development. The egg samples were incubated with $NaHC^{14}O_3$ for $3\,^1/_2$ hours. Ordinate: counts per minute per 15 mg $BaCO_3$ per cm^2.

2) Rates of isotope incorporation into the protein fraction (carboxyl C) in the same experiments.

3) Relative respiration rate of sea urchin embryos at corresponding stages of development. Full line: *Psammechinus miliaris*, recalculated from Borei (1). Broken line: *Paracentrotus lividus*, according to Lindahl (12).

Fig. 2. 4) The isotope incorporation rates of the proteins (carboxyl C) at different stages referred to the corresponding values of the TCA-soluble fraction (total C).

5) The isotope incorporation rates of the soluble proteins at different stages related to the corresponding incorporation rates of the non-soluble proteins.

Hatching at $7\,^1/_4$ hours of development. Primary mesenchyme cells appeared at 10 hours. Invagination started at 13 hours.

Results and discussion: The rate of isotope uptake into the TCA-soluble fraction increased progressively during the cleavage stages, reached a maximum about the time of hatching, and then decreased continuously from a stage shortly before the appearance of the primary mesenchyme cells. The specific activity of the protein carboxyl carbon increased shortly after fertilization and attained a maximum simultaneously with the TCA-soluble fraction. At the beginning of gastrulation, a second increase was observed.

It is not believed that the carbon dioxide uptake into sea urchin embryos at different stages of development is fundamentally influenced by permeability factors. However, the intensity of respiration of the embryos changes during development (3, 12, 1), cf. Fig. 1. The uptake of labeled carbon dioxide into the TCA-soluble fraction (Fig. 1) increased approximately in proportion to the respiration during the first respiratory phase, while a decline was noted during the second period. It is obvious that the isotope added for the incubations became diluted by respiratory carbon dioxide in proportion to the respiratory level. The extent of this dilution did not lend itself to direct measurements in the present experiments. By comparison of our data, in reference to equal amounts of nitrogen, with the respiration measurements mentioned above, it became evident that the isotope dilution effect contributed to the decrease in specific activity observed in the TCA-soluble fraction during the second phase of respiration. However, the pronounced change in isotope uptake between the first and second phases of respiration could not be explained exclusively

as an effect of isotope dilution. It seems probable that this change is an expression of alterations in the catabolic pattern during development (cf. 12). The important role played by certain acids of the tricarboxylic acid cycle in carbon fixation in animal tissues is well known (20). It has been shown recently that the second respiratory phase in the sea urchin development is accompanied by an increased activity of tricarboxylic acid cycle enzymes (5). However, the actual extent of carbon dioxide fixation in a cell does not depend solely upon the enzymatic complement but also on conditions influencing the reaction equilibria at the initial steps of carboxylation. As examples of such conditions, one might mention the energetic level (19), the degree of enzyme saturation (cf. 4), and the degree of reduction of the triphosphopyridine nucleotide (TPN) (15, 16). For the reduction of TPN the enzymatic oxidation of glucose-6-phosphate, by way of 6-phosphogluconic acid is important (15, 16, cf. 2). This path of carbohydrate degradation has been demonstrated in sea urchin material (13). It seems very plausible that its relative importance is elevated during the culmination period of the first respiratory phase, before the increased capacity of the tricarboxylic acid cycle.

By referring the specific activities of the protein carboxyl groups to those of the TCA-soluble total carbon it is possible to approximately compensate for the variations in isotope dilution and carbon dioxide fixation rate during development. These recalculated protein values (Fig. 2) show a continuous acceleration of isotope incorporation after the mesenchyme blastula stage. This sharp increase may be due to an accelerated isotope uptake into amino acids or to an increased rate of incorporation of labeled amino acids into proteins. It was shown previously (10) that the incorporation of N^{15}-labeled glycine or alanine into the water-soluble proteins of developing embryos was accelerated during the blastula stage. However, the incorporation into the non-soluble proteins increased soon after fertilization, and the total protein fraction in such experiments does not show the sharp increase during the late blastula stage seen in the present experiments. It will be shown in a later report that the uptake of N^{15}-labeled ammonia into amino acids is much accelerated from the blastula stage on. The marked increase during the late blastula stage in the present experiments is therefore interpreted as due to an increased metabolic rate largely at the amino acid level (cf. 11, 6).

The isotope incorporation was higher in the non-soluble than in the soluble proteins. From the blastula stage on, however, the incorporation into the soluble proteins became intensified (Fig. 2). In this respect, the present experiments are quite similar to those with glycine, alanine and ammonia, reported previously (9, 10).

The general picture of the isotope uptake at the two different pH-levels was very similar, but all specific activities were considerably lower at the higher pH.

REFERENCES

1. Borei, H., *Biol. Bull.*, **95**, 124 (1948).
2. Crane, R. K., and Ball, E. G., *J. Biol. Chem.*, **189**, 269 (1951).
3. Gray, J., *Brit. J. Exptl. Biol.*, **4**, 313 (1926).
4. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172**, 389 (1948).
5. Gustafson, T., and Hasselberg, I., *Exptl. Cell Res.*, **2** 642 (1951).
6. Gustafson, T., and Hjelte, M. B., *ibid.*, **2**, 474 (1951).

7. Hultin, T., *ibid.*, **1**, 159 (1950).
8. —— *ibid.*, **1**, 272 (1950).
9. —— *ibid.*, **1**, 599 (1950).
10. —— *ibid.*, **3**, 494 (1952).
11. Lindahl, P. E., *Acta Zool.*, **17**, 179 (1936).
12. —— *Z. vergl. Physiol.*, **27**, 233 (1939).
13. Lindberg, O., *Arkiv Kemi, Mineral., Geol.*, **16** *A*, No. 15 (1943).
14. Monné, L., and Slautterback, D. B., *Exptl. Cell Res.*, **1**, 477 (1950).
15. Ochoa, S., *J. Biol. Chem.*, **174**, 133 (1948).
16. Ochoa, S., Mehler, A. H., and Kornberg, A., *J. Biol. Chem.*, **174**, 979 (1948).
17. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
18. Tyler, A., and Horowitz, N. H., *Science*, **86**, 85 (1937).
19. Utter, M. F., and Wood, H. G., *J. Biol. Chem.*, **164**, 455 (1946).
20. —— *Advances in Enzymol.*, **12**, 41 (1951).
21. Van Slyke, D. D., MacFayden, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).

ALKALINE PHOSPHATASE ACTIVITY IN THE GOLGI SUBSTANCE OF INTESTINAL MUCOSA[1]

ALEX B. NOVIKOFF, LORRAINE KORSON and HERMAN W. SPATER

Departments of Pathology and Oncology and of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont

Received April 2, 1952

In a recent review, Bensley (1) has written that although the association of alkaline phosphatase and the Golgi substance is a "highly plausible assumption," he "does not regard the evidence so far adduced as conclusive." The evidence has consisted of the strongly positive staining reaction given by this substance in the Gomori-Takamatsu histochemical technique. Such staining was shown to be artefactual in the case of cell nuclei by Martin and Jacoby (6) and by several other investigators (2, 10, 9, 5) who established that calcium phosphate was preferentially adsorbed by nuclei. As suggested by Martin and Jacoby (6), there remained the possibility that the staining of the Golgi zone was due to a similar cause. We are now reporting observations which indicate that this is not the case; rather, alkaline phosphatase activity appears to be one of the intrinsic properties of the Golgi substance.

Small pieces of mouse and rat duodenum were treated in the following fashion: 1) Some were fixed in ice-cold acetone for 18—24 hours, passed through absolute alcohol (18–24 hours) and benzene (2 hours), and then embedded in paraffin (infiltration at 56° C. for 40 min., *in vacuo*), and 2) Others were instantly frozen in isopentane at —160° C., dehydrated *in vacuo* at —42° C. for 12 hours and embedded in paraffin (infiltration at 56° C. for 20 min., *in vacuo*). Sections (6 μ) were treated by two methods for demonstrating alkaline phosphatase activity, the Gomori-Takamatsu calcium phosphate-cobalt sulfide procedure (4) and the azo-dye method of Menten *et al.* (7) as modified by Gomori (5).

With the Gomori-Takamatsu method, very short incubation periods, of the order of 45–75 seconds, are sufficient to visualize the Golgi substance in both the fixed and frozen-dried material (Fig. 1 A, B). By this time the striated border is already quite dark and it is thus conceivable that the staining of the Golgi substance is due to the adsorption of calcium phosphate diffused from the striated border. This seems somewhat unlikely since the nuclei, despite their high affinity for calcium phosphate, are completely unstained, and other experiments make this possibility appear quite remote. These were done with sections in which enzyme activity was completely destroyed by treatment with distilled water at 90° C. for 5 minutes. Such inactivated sections were incubated in the usual substrate medium to which was added either purified soluble alkaline phosphatase or dilute hydrogen peroxide, in order to produce calcium phosphate. They were then processed in the usual manner, with the results shown in Fig. 1 D. The nuclei and striated border, having

[1] This work was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

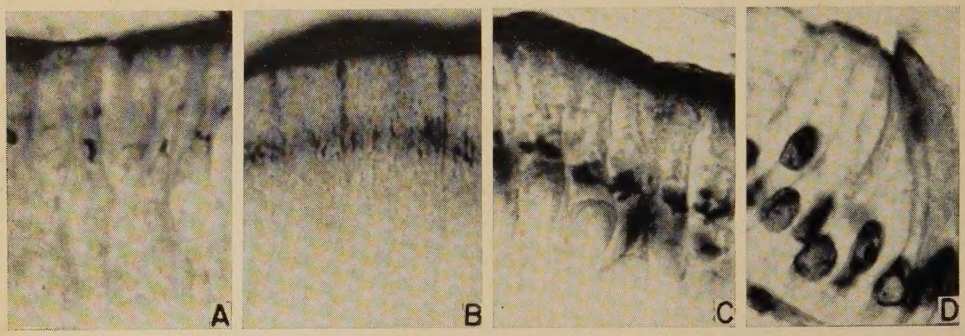


Fig. 1. All photographs were taken with a Wratten B No. 58 green filter. Magnification = 1 140 ×.
A. Mouse duodenum; acetone-fixed; incubated for 75 sec. at 37° C. in the following medium (pH 9.4): 1 *M* 2-amino-2-methyl-1,3-propanediol, 50 ml, 2 per cent sodium β glycerophosphate (Mallinckrodt), 25 ml; 2 per cent calcium chloride, 5 ml; 2 per cent magnesium sulfate, 2 ml; distilled water 25 ml.
B. Rat duodenum; frozen-dried; incubated for 60 sec., as in Fig. 1 A.
C. Mouse duodenum; acetone fixed; incubated for 20 min. at 14–18° C. in the following medium after filtration: 0.05 per cent sodium α naphthyl acid phosphate, 40 ml, 5 per cent borax, 10 ml; 3 drops, 0.5 *M* magnesium chloride; 25 mg. diazotized 4-Cl-2-aminoanisole.
D. Mouse duodenum; acetone-fixed; inactivated. Calcium phosphate produced by addition of 0.4 ml 3 per cent H_2O_2 to 40 ml of incubation medium given in Fig. 1 A.

adsorbed the phosphate, are stained, but there is no sign of adsorption by the Golgi substance.

With the azo dye method, only structures of high alkaline phosphatase activity would be expected to stain (see 9) and, indeed, the striated border and Golgi substance are the only positive structures (Fig. 1 C). Again, inactivated sections were incubated in the substrate medium to which was added soluble alkaline phosphatase. The azo dye produced was not adsorbed by the Golgi substance or by any other material in the section.

There is still the possibility that *enzyme* of the striated border has diffused to and been adsorbed by the Golgi substance. This is quite unlikely since: 1) as we have seen and as Emmel (3) has reported earlier, instantly-frozen unfixed tissue shows enzyme activity in the Golgi substance, and 2) Moog (8) has reported that even after 30 minutes of incubation in the Gomori medium at 38° C., there is no evidence of decreased activity in the striated border.

REFERENCES

1. Bensley, R. R., *Exptl. Cell Res.*, **2**, 1 (1951).
2. Cleland, K. W., *Proc. Linnean Soc., N. S. Wales*, **75**, 54 (1950).
3. Emmel, V. M., *Anat. Rec.*, **95**, 159 (1946).
4. Gomori, G., *Proc. Soc. Exptl. Biol. Med.*, **42**, 23 (1939).
5. —— *J. Lab. Clin. Med.*, **37**, 526 (1951).
6. Martin, B. F., and Jacoby, F., *J. Anat.*, **83**, 351 (1949).
7. Menten, M. L., Junge, J., and Green, M. H., *J. Biol. Chem.*, **153**, 471 (1944).
8. Moog, F., *Science*, **114**, 109 (1951).
9. Novikoff, A. B., *Science*, **113**, 320 (1951).
10. Yokoyama, H. O., Stowell, R. E., and Mathews, R. M., *Anat. Rec.*, **109**, 139 (1951).